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TITLE OF THE INVENTION (500 characters maximum)

ANTAGONIST ANTI-CD40 MONOCLONAL ANTIBODIES AND METHODS
FOR THEIR USE

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- ☐ Application Data Sheet. See 37 CFR 1.76
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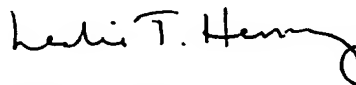
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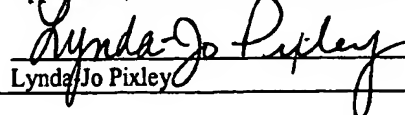


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ANTAGONIST ANTI-CD40 MONOCLONAL ANTIBODIES AND
METHODS FOR THEIR USE

FIELD OF THE INVENTION

The invention relates to human antibodies capable of binding to CD40, methods of using the antibodies, and methods for treatment of diseases mediated by stimulation of CD40 signaling on CD40-expressing cells.

BACKGROUND OF THE INVENTION

B cells play an important role during the normal *in vivo* immune response. A foreign antigen will bind to surface immunoglobulins on specific B cells, triggering a chain of events including endocytosis, processing, presentation of processed peptides on MHC-class II molecules, and up-regulation of the B7 antigen on the B cell surface. A specific T cell then binds to the B cell via T cell receptor (TCR) recognition of the processed antigen presented on the MHC-class II molecule. Stimulation through the TCR activates the T cell and initiates T-cell cytokine production. A second signal that further activates the T cell is an interaction between the CD28 antigen on T cells and the B7 antigen on B cells. When the above-mentioned signals are received, the CD40 ligand (CD40L or CD154), which is not expressed on resting human T cells, is up-regulated on the T-cell surface. Binding of the CD40 ligand to the CD40 antigen on the B cell surface stimulates the B cell, causing the B cell to mature into a plasma cell secreting high levels of soluble immunoglobulin.

CD40 is a 55 kDa cell-surface antigen present on the surface of both normal and neoplastic human B cells, dendritic cells, antigen presenting cells (APCs), endothelial cells, monocytic and epithelial cells. Transformed cells from patients with low- and high-grade B-cell lymphomas, B-cell acute lymphoblastic leukemia, multiple myeloma,

chronic lymphocytic leukemia, and Hodgkin's disease express CD40. CD40 expression is also detected in two-thirds of acute myeloblastic leukemia cases and 50% of AIDS-related lymphomas. Malignant B cells from several tumors of B-cell lineage express a high degree of CD40 and appear to depend on CD40 signaling for survival and proliferation.

Immunoblastic B-cell lymphomas frequently arise in immunocompromised individuals such as allograft recipients and others receiving long-term immunosuppressive therapy, AIDS patients, and patients with primary immunodeficiency syndromes such as X-linked lymphoproliferative syndrome or Wiscott-Aldrich syndrome (Thomas *et al.* (1991) *Adv. Cancer Res.* 57:329; Straus *et al.* (1993) *Ann. Intern. Med.* 118:45).

The CD40 antigen is related to the human nerve growth factor (NGF) receptor, tumor necrosis factor- α (TNF- α) receptor, and Fas, suggesting that CD40 is a receptor for a ligand with important functions in B-cell activation. CD40 expression on APCs plays an important co-stimulatory role in the activation of both T-helper and cytotoxic T lymphocytes. The CD40 receptor is expressed on activated T cells, activated platelets, and inflamed vascular smooth muscle cells. CD40 receptors can also be found on eosinophils, synovial membranes in rheumatoid arthritis, dermal fibroblasts, and other non-lymphoid cell types. Binding of CD40L to the CD40 receptor stimulates B-cell proliferation and differentiation, antibody production, isotype switching, and B-cell memory generation.

BRIEF SUMMARY OF THE INVENTION

Compositions and methods are provided for treating diseases mediated by stimulation of CD40 signaling on CD40-expressing cells, including lymphomas, autoimmune diseases, and transplant rejections. Compositions include monoclonal antibodies capable of binding to a human CD40 antigen located on the surface of a human CD40-expressing cell, wherein the binding prevents the growth or differentiation of the cell. Compositions also include monoclonal antibodies capable of specifically binding to a human CD40 antigen expressed on the surface of a human CD40-expressing cell, said monoclonal antibody being free of significant agonistic activity, wherein

administration of said monoclonal antibody results in significantly less tumor volume than a similar concentration of the chimeric anti-CD20 monoclonal antibody IDEC-C2B8 in a staged nude mouse xenograft tumor model using the Daudi human B cell lymphoma cell line. Compositions also include antigen-binding fragments of these monoclonal antibodies, hybridoma cell lines producing these antibodies, and isolated nucleic acid molecules encoding the amino acid sequences of these monoclonal antibodies. The invention further includes pharmaceutical compositions comprising the such anti-CD40 antibodies in a pharmaceutically acceptable carrier.

Methods are provided for preventing or treating a disease mediated by stimulation of CD40 signaling, comprising treating the patient with an anti-CD40 antibody or an antigen-binding fragment thereof that is free of significant agonist activity when bound to a CD40 antigen on a human CD40-expressing cell. Diseases mediated by stimulation of CD40-expressing cells include autoimmune diseases, cancers, and organ and tissue graft rejections. Lymphomas that can be treated or prevented by a method of the present invention include non-Hodgkin's lymphomas (high-grade lymphomas, intermediate-grade lymphomas, and low-grade lymphomas), Hodgkin's disease, acute lymphoblastic leukemias, myelomas, chronic lymphocytic leukemias, and myeloblastic leukemias.

Particular autoimmune diseases contemplated for treatment using the methods of the invention include systemic lupus erythematosus (SLE), rheumatoid arthritis, Crohn's disease, psoriasis, autoimmune thrombocytopenic purpura, multiple sclerosis, ankylosing spondylitis, myasthenia gravis, and pemphigus vulgaris. Such antibodies could also be used to prevent rejection of organ and tissue grafts by suppressing autoimmune responses, to treat lymphomas by depriving malignant B lymphocytes of the activating signal provided by CD40, and to deliver toxins to CD40-bearing cells in a specific manner.

Methods for inhibiting the growth, differentiation, and/or proliferation of human B cells and for inhibiting antibody production by B cells in a human patient are provided, as are methods for inhibiting the growth of cancer cells of a B-cell lineage. Methods for identifying antibodies that have antagonistic activity toward CD40-expressing cells are also provided.

The monoclonal antibodies disclosed herein have a strong affinity for CD40 and are characterized by a dissociation equilibrium constant (K_D) of at least 10^{-6} M, preferably at least about 10^{-7} M to about 10^{-8} M, more preferably at least about 10^{-8} M to about 10^{-12} M. Monoclonal antibodies and antigen-binding fragments thereof that are

5 suitable for use in the methods of the invention are capable of specifically binding to a human CD40 antigen expressed on the surface of a human cell. They are free of significant agonist activity but exhibit antagonist activity when bound to CD40 antigen on human cells. In one embodiment, the anti-CD40 antibody or fragment thereof exhibits antagonist activity when bound to CD40 antigen on normal human B cells. In another

10 embodiment, the anti-CD40 antibody or fragment thereof exhibits antagonist activity when bound to CD40 antigen on malignant human B cells. Suitable monoclonal antibodies have human constant regions; preferably they also have wholly or partially humanized framework regions; and most preferably are fully human antibodies or antigen-binding fragments thereof. Examples of such monoclonal antibodies are the

15 antibodies designated herein as 5.9 and 12.12; the monoclonal antibodies produced by the hybridoma cell lines designated 131.2F8.5.9 (referred to herein as the cell line 5.9) and 153.8E2.D10.D6.12.12 (referred to herein as the cell line 12.12); a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:6, the sequence shown in SEQ ID NO:7, the sequence shown in

20 SEQ ID NO:8, both the sequence shown in SEQ ID NO:6 and SEQ ID NO:7, and both the sequence shown in SEQ ID NO:6 and SEQ ID NO:8; a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:2, the sequence shown in SEQ ID NO:4, the sequence shown in SEQ ID NO:5, both the sequence shown in SEQ ID NO:2 and SEQ ID NO:4, and both

25 the sequence shown in SEQ ID NO:2 and SEQ ID NO:5; a monoclonal antibody comprising an amino acid sequence encoded by a nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of the sequence shown in SEQ ID NO:1, the sequence shown in SEQ ID NO:3, and both the sequence shown in SEQ ID NO:1 and SEQ ID NO:3; and antigen-binding fragments of these monoclonal antibodies

30 that retain the capability of specifically binding to human CD40, and which are free of significant agonist activity but exhibit antagonist activity when bound to CD40 antigen

on human cells. Examples of such monoclonal antibodies also include a monoclonal antibody that binds to an epitope capable of binding the monoclonal antibody produced by the hybridoma cell line 12.12; a monoclonal antibody that binds to an epitope comprising residues 82-87 of the amino acid sequence shown in SEQ ID NO:10 or SEQ ID NO:12; a monoclonal antibody that competes with the monoclonal antibody 12.12 in a competitive binding assay; and a monoclonal antibody that is an antigen-binding fragment of the 12.12 monoclonal antibody or any of the foregoing monoclonal antibodies, where the fragment retains the capability of specifically binding to the human CD40 antigen.

In one embodiment of the invention, methods of treatment comprise administering to a patient a therapeutically effective dose of a pharmaceutical composition comprising suitable antagonistic anti-CD40 antibodies or antigen-binding fragments thereof. A therapeutically effective dose of the anti-CD40 antibody or fragment thereof is in the range from about 0.01 mg/kg to about 40 mg/kg, from about 0.01 mg/kg to about 30 mg/kg, from about 0.1 mg/kg to about 30 mg/kg, from about 1 mg/kg to about 30 mg/kg, from about 3 mg/kg to about 30 mg/kg, from about 3 mg/kg to about 25 mg/kg, from about 3 mg/kg to about 20 mg/kg, from about 5 mg/kg to about 15 mg/kg, or from about 7 mg/kg to about 12 mg/kg. It is recognized that the method of treatment may comprise a single administration of a therapeutically effective dose or multiple administrations of a therapeutically effective dose of the antagonist anti-CD40 antibody or antigen-binding fragment thereof.

The antagonist anti-CD40 antibodies identified herein as being suitable for use in the methods of the invention may be modified. Modifications of these antagonist anti-CD40 antibodies include, but are not limited to, immunologically active chimeric anti-CD40 antibodies, humanized anti-CD40 antibodies, and immunologically active murine anti-CD40 antibodies.

The following embodiments are encompassed by the present invention:

1. A human monoclonal antibody that is capable of specifically binding to a human CD40 antigen expressed on the surface of a human CD40-expressing cell, said monoclonal antibody being free of significant agonistic activity, wherein said monoclonal antibody exhibits increased anti-tumor activity relative to an equivalent amount of the

monoclonal chimeric anti-CD20 monoclonal antibody IDEC-C2B8, wherein said anti-tumor activity is assayed in a staged nude mouse xenograft tumor model using the Daudi human B cell lymphoma cell line.

5 2. The human monoclonal antibody of embodiment 1, wherein said antibody is selected from the group consisting of:

 a) the monoclonal antibody 12.12;

 b) the monoclonal antibody produced by the hybridoma cell line 12.12, deposited with the ATCC as Patent Deposit No. PTA-5543;

10 c) a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:2, the sequence shown in SEQ ID NO:4, the sequence shown in SEQ ID NO:5, both the sequence shown in SEQ ID NO:2 and SEQ ID NO:4, and both the sequence shown in SEQ ID NO:2 and SEQ ID NO:5;

15 d) a monoclonal antibody having an amino acid sequence encoded by a nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of the sequence shown in SEQ ID NO:1, the sequence shown in SEQ ID NO:3, and both the sequence shown in SEQ ID NO:1 and SEQ ID NO:3;

 e) a monoclonal antibody that binds to an epitope capable of binding the
20 monoclonal antibody produced by the hybridoma cell line 12.12;

 f) a monoclonal antibody that binds to an epitope comprising residues 82-87 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12;

 g) a monoclonal antibody that competes with the monoclonal antibody 12.12 in a competitive binding assay; and,

25 h) a monoclonal antibody that is an antigen-binding fragment of a monoclonal antibody of a)-g), wherein said fragment retains the capability of specifically binding to said human CD40 antigen.

 3. The monoclonal antibody of embodiment 1, wherein said monoclonal
30 antibody binds to said human CD40 antigen with an affinity (K_D) of at least about 10^{-6} M to about 10^{-12} M.

4. A hybridoma cell line capable of producing the monoclonal antibody of embodiment 1.

5. A method for treating a cancer characterized by expression of CD40, comprising administering to a human patient an effective amount of a human anti-CD40 monoclonal antibody of embodiment 1.

6. The method of embodiment 5, wherein said cancer is selected from the group consisting of a non-Hodgkins lymphoma, chronic lymphocytic leukemia, multiple myeloma, B cell lymphoma, high-grade B cell lymphoma, intermediate-grade B cell lymphoma, low-grade B cell lymphoma, B cell acute lymphoblastic leukemia, myeloblastic leukemia, and Hodgkin's disease.

7. A human monoclonal antibody that is capable of specifically binding to a human CD40 antigen expressed on the surface of a human CD40-expressing cell, said monoclonal antibody being free of significant agonistic activity, whereby, when said monoclonal antibody binds to the CD40 antigen expressed on the surface of said cell, the growth or differentiation of said cell is inhibited, wherein said antibody is selected from the group consisting of:

a) the monoclonal antibody 5.9 or 12.12;

b) the monoclonal antibody produced by the hybridoma cell line 5.9 or 12.12;

c) a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:6, the sequence shown in SEQ ID NO:7, the sequence shown in SEQ ID NO:8, both the sequence shown in SEQ ID NO:6 and SEQ ID NO:7, and both the sequence shown in SEQ ID NO:6 and SEQ ID NO:8;

d) a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:2, the sequence shown in SEQ ID NO:4, the sequence shown in SEQ ID NO:5, both the sequence shown in SEQ

ID NO:2 and SEQ ID NO:4, and both the sequence shown in SEQ ID NO:2 and SEQ ID NO:5;

e) a monoclonal antibody having an amino acid sequence encoded by a nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of the sequence shown in SEQ ID NO:1, the sequence shown in SEQ ID NO:3, and both the sequence shown in SEQ ID NO:1 and SEQ ID NO:3;

f) a monoclonal antibody that binds to an epitope capable of binding the monoclonal antibody produced by the hybridoma cell line 5.9 or 12.12;

g) a monoclonal antibody that binds to an epitope comprising residues 82-87 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12;

h) a monoclonal antibody that binds to an epitope comprising residues 82-89 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12;

i) a monoclonal antibody that competes with the monoclonal antibody 5.9 or 12.12 in a competitive binding assay; and,

j) a monoclonal antibody that is an antigen-binding fragment of a monoclonal antibody of a)-i), wherein said fragment retains the capability of specifically binding to said human CD40 antigen.

8. The antigen-binding fragment of embodiment 7, wherein said fragment is selected from the group consisting of a Fab fragment, an $F(ab')_2$ fragment, an Fv fragment, and a single-chain Fv fragment.

9. The monoclonal antibody of embodiment 7, wherein said monoclonal antibody binds to said human CD40 antigen with an affinity (K_D) of at least about 10^{-6} M to about 10^{-12} M.

10. An isolated nucleic acid molecule comprising a polynucleotide that encodes an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:8.

11. A hybridoma cell line capable of producing a human monoclonal antibody having specificity for a human CD40 antigen expressed on the surface of a human CD40-expressing cell, whereby said monoclonal antibody is free of significant agonistic activity, whereby, when said monoclonal antibody binds to the CD40 antigen expressed on the surface of said cell, the growth or differentiation of said cell is inhibited, and wherein said monoclonal antibody is selected from the group consisting of:

a) the monoclonal antibody 5.9 or 12.12;
b) the monoclonal antibody produced by the hybridoma cell line 5.9 or 12.12;

c) a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:6, the sequence shown in SEQ ID NO:7, the sequence shown in SEQ ID NO:8, both the sequence shown in SEQ ID NO:6 and SEQ ID NO:7, and both the sequence shown in SEQ ID NO:6 and SEQ ID NO:8;

d) a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:2, the sequence shown in SEQ ID NO:4, the sequence shown in SEQ ID NO:5, both the sequence shown in SEQ ID NO:2 and SEQ ID NO:4, and both the sequence shown in SEQ ID NO:2 and SEQ ID NO:5;

e) a monoclonal antibody having an amino acid sequence encoded by a nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of the sequence shown in SEQ ID NO:1, the sequence shown in SEQ ID NO:3, and both the sequence shown in SEQ ID NO:1 and SEQ ID NO:3;

f) a monoclonal antibody that binds to an epitope capable of binding the monoclonal antibody produced by the hybridoma cell line 5.9 or 12.12;

g) a monoclonal antibody that binds to an epitope comprising residues 82-87 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12;

h) a monoclonal antibody that binds to an epitope comprising residues 82-89 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12;

i) a monoclonal antibody that competes with the monoclonal antibody 5.9 or 12.12 in a competitive binding assay; and,

j) a monoclonal antibody that is an antigen-binding fragment of a monoclonal antibody of a)-i), wherein said fragment retains the capability of specifically binding to said human CD40 antigen.

5 12. A method for inhibiting growth or differentiation of a normal human B cell, comprising contacting said B cell with an effective amount of a human anti-CD40 monoclonal antibody selected from the group consisting of:

- a) the monoclonal antibody 5.9 or 12.12;
- b) the monoclonal antibody produced by the hybridoma cell line 5.9 or

10 12.12;

c) a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:6, the sequence shown in SEQ ID NO:7, the sequence shown in SEQ ID NO:8, both the sequence shown in SEQ ID NO:6 and SEQ ID NO:7, and both the sequence shown in SEQ ID NO:6 and SEQ ID

15 NO:8;

d) a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:2, the sequence shown in SEQ ID NO:4, the sequence shown in SEQ ID NO:5, both the sequence shown in SEQ ID NO:2 and SEQ ID NO:4, and both the sequence shown in SEQ ID NO:2 and SEQ ID

20 NO:5;

e) a monoclonal antibody having an amino acid sequence encoded by a nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of the sequence shown in SEQ ID NO:1, the sequence shown in SEQ ID NO:3, and both the sequence shown in SEQ ID NO:1 and SEQ ID NO:3;

25 f) a monoclonal antibody that binds to an epitope capable of binding the monoclonal antibody produced by the hybridoma cell line 5.9 or 12.12;

g) a monoclonal antibody that binds to an epitope comprising residues 82-87 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12;

h) a monoclonal antibody that binds to an epitope comprising residues 82-89

30 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12;

i) a monoclonal antibody that competes with the monoclonal antibody 5.9 or 12.12 in a competitive binding assay; and,

j) a monoclonal antibody that is an antigen-binding fragment of a monoclonal antibody of a)-i), wherein said fragment retains the capability of specifically binding to said human CD40 antigen; said antibody or fragment thereof being free of significant agonistic activity, and whereby when said antibody or fragment thereof binds to said CD40 antigen on said B cell, the growth or differentiation of said B cell is inhibited.

13. The method of embodiment 12, wherein said monoclonal antibody or fragment thereof binds to said human CD40 antigen with an affinity (K_D) of at least about 10^{-6} M to about 10^{-12} M.

14. The method of embodiment 12, wherein said fragment is selected from the group consisting of a Fab fragment, an $F(ab')_2$ fragment, an Fv fragment, and a single-chain Fv fragment.

15. A method for inhibiting proliferation of a normal human B cell, wherein said proliferation is augmented by the interaction of a CD40 ligand with a CD40 antigen expressed on the surface of said B cell, said method comprising contacting said B cell with an effective amount of a human anti-CD40 monoclonal antibody selected from the group consisting of:

- a) the monoclonal antibody 5.9 or 12.12;
- b) the monoclonal antibody produced by the hybridoma cell line 5.9 or

12.12;

c) a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:6, the sequence shown in SEQ ID NO:7, the sequence shown in SEQ ID NO:8, both the sequence shown in SEQ ID NO:6 and SEQ ID NO:7, and both the sequence shown in SEQ ID NO:6 and SEQ ID NO:8;

d) a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:2, the sequence shown in SEQ ID NO:4, the sequence shown in SEQ ID NO:5, both the sequence shown in SEQ ID NO:2 and SEQ ID NO:4, and both the sequence shown in SEQ ID NO:2 and SEQ ID NO:5;

e) a monoclonal antibody having an amino acid sequence encoded by a nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of the sequence shown in SEQ ID NO:1, the sequence shown in SEQ ID NO:3, and both the sequence shown in SEQ ID NO:1 and SEQ ID NO:3;

f) a monoclonal antibody that binds to an epitope capable of binding the monoclonal antibody produced by the hybridoma cell line 5.9 or 12.12;

g) a monoclonal antibody that binds to an epitope comprising residues 82-87 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12;

h) a monoclonal antibody that binds to an epitope comprising residues 82-89 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12;

i) a monoclonal antibody that competes with the monoclonal antibody 5.9 or 12.12 in a competitive binding assay; and,

j) a monoclonal antibody that is an antigen-binding fragment of a monoclonal antibody of a)-i), wherein said fragment retains the capability of specifically binding to said human CD40 antigen; said antibody or fragment thereof being free of significant agonistic activity, and whereby when said antibody or fragment thereof binds to said CD40 antigen on said B cell, the growth or differentiation of said B cell is inhibited.

16. The method of embodiment 15, wherein said monoclonal antibody binds to said human CD40 antigen with an affinity (K_D) of at least about 10^{-6} M to about 10^{-12} M.

17. The method of embodiment 15, wherein said fragment is selected from the group consisting of a Fab fragment, an $F(ab')_2$ fragment, an Fv fragment, and a single-chain Fv fragment.

18. A method for inhibiting antibody production by B cells in a human patient, comprising administering to a human patient an effective amount of a human anti-CD40 monoclonal antibody or fragment thereof selected from the group consisting
5 of:

a) the monoclonal antibody 5.9 or 12.12;

b) the monoclonal antibody produced by the hybridoma cell line 5.9 or 12.12;

c) a monoclonal antibody comprising an amino acid sequence selected from
10 the group consisting of the sequence shown in SEQ ID NO:6, the sequence shown in SEQ ID NO:7, the sequence shown in SEQ ID NO:8, both the sequence shown in SEQ ID NO:6 and SEQ ID NO:7, and both the sequence shown in SEQ ID NO:6 and SEQ ID NO:8;

d) a monoclonal antibody comprising an amino acid sequence selected from
15 the group consisting of the sequence shown in SEQ ID NO:2, the sequence shown in SEQ ID NO:4, the sequence shown in SEQ ID NO:5, both the sequence shown in SEQ ID NO:2 and SEQ ID NO:4, and both the sequence shown in SEQ ID NO:2 and SEQ ID NO:5;

e) a monoclonal antibody having an amino acid sequence encoded by a
20 nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of the sequence shown in SEQ ID NO:1, the sequence shown in SEQ ID NO:3, and both the sequence shown in SEQ ID NO:1 and SEQ ID NO:3;

f) a monoclonal antibody that binds to an epitope capable of binding the monoclonal antibody produced by the hybridoma cell line 5.9 or 12.12;

25 g) a monoclonal antibody that binds to an epitope comprising residues 82-87 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12;

h) a monoclonal antibody that binds to an epitope comprising residues 82-89 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12;

i) a monoclonal antibody that competes with the monoclonal antibody 5.9 or
30 12.12 in a competitive binding assay; and,

j) a monoclonal antibody that is an antigen-binding fragment of a monoclonal antibody of a)-i), wherein said fragment retains the capability of specifically binding to said human CD40 antigen;
said antibody or fragment thereof being free of significant agonistic activity, and whereby
5 when said antibody or fragment thereof binds to said CD40 antigen on said B cell, the growth or differentiation of said B cell is inhibited.

19. The method of embodiment 18, wherein said monoclonal antibody binds to said human CD40 antigen with an affinity (K_D) of at least about 10^{-6} M to about 10^{-12}
10 M.

20. The method of embodiment 18, wherein said fragment is selected from the group consisting of a Fab fragment, an $F(ab')_2$ fragment, an Fv fragment, and a single-chain Fv fragment.

15 21. A method for inhibiting growth of cancer cells of B cell lineage, comprising contacting said cancer cells with an effective amount of a human anti-CD40 monoclonal antibody selected from the group consisting of:

- a) the monoclonal antibody 5.9 or 12.12;
- 20 b) the monoclonal antibody produced by the hybridoma cell line 5.9 or 12.12;
- c) a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:6, the sequence shown in SEQ ID NO:7, the sequence shown in SEQ ID NO:8, both the sequence shown in SEQ
25 ID NO:6 and SEQ ID NO:7, and both the sequence shown in SEQ ID NO:6 and SEQ ID NO:8;
- d) a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:2, the sequence shown in SEQ ID NO:4, the sequence shown in SEQ ID NO:5, both the sequence shown in SEQ
30 ID NO:2 and SEQ ID NO:4, and both the sequence shown in SEQ ID NO:2 and SEQ ID NO:5;

e) a monoclonal antibody having an amino acid sequence encoded by a nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of the sequence shown in SEQ ID NO:1, the sequence shown in SEQ ID NO:3, and both the sequence shown in SEQ ID NO:1 and SEQ ID NO:3;

5 f) a monoclonal antibody that binds to an epitope capable of binding the monoclonal antibody produced by the hybridoma cell line 5.9 or 12.12;

g) a monoclonal antibody that binds to an epitope comprising residues 82-87 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12;

10 h) a monoclonal antibody that binds to an epitope comprising residues 82-89 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12;

i) a monoclonal antibody that competes with the monoclonal antibody 5.9 or 12.12 in a competitive binding assay; and,

15 j) a monoclonal antibody that is an antigen-binding fragment of a monoclonal antibody of a)-i), wherein said fragment retains the capability of specifically binding to said human CD40 antigen;
said antibody or fragment thereof being free of significant agonistic activity, and whereby when said antibody or fragment thereof binds to said CD40 antigen on said B cell, the growth or differentiation of said B cell is inhibited.

20 22. The method of embodiment 21, wherein said monoclonal antibody binds to said human CD40 antigen with an affinity (K_D) of at least about 10^{-6} M to about 10^{-12} M.

25 23. The method of embodiment 21, wherein said fragment is selected from the group consisting of a Fab fragment, an $F(ab')_2$ fragment, an Fv fragment, and a single-chain Fv fragment.

30 24. The method of embodiment 21, wherein the cancer is selected from the group consisting of non-Hodgkins lymphoma, chronic lymphocytic leukemia, multiple myeloma, B cell lymphoma, high-grade B cell lymphoma, intermediate-grade B cell

lymphoma, low-grade B cell lymphoma, B cell acute lymphoblastic leukemia, myeloblastic leukemia, and Hodgkin's disease.

25. A method for treating an autoimmune disease, comprising administering
5 to a human patient an effective amount of a human anti-CD40 monoclonal antibody selected from the group consisting of:

- a) the monoclonal antibody 5.9 or 12.12;
- b) the monoclonal antibody produced by the hybridoma cell line 5.9 or 12.12;

10 c) a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:6, the sequence shown in SEQ ID NO:7, the sequence shown in SEQ ID NO:8, both the sequence shown in SEQ ID NO:6 and SEQ ID NO:7, and both the sequence shown in SEQ ID NO:6 and SEQ ID NO:8;

15 d) a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:2, the sequence shown in SEQ ID NO:4, the sequence shown in SEQ ID NO:5, both the sequence shown in SEQ ID NO:2 and SEQ ID NO:4, and both the sequence shown in SEQ ID NO:2 and SEQ ID NO:5;

20 e) a monoclonal antibody having an amino acid sequence encoded by a nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of the sequence shown in SEQ ID NO:1, the sequence shown in SEQ ID NO:3, and both the sequence shown in SEQ ID NO:1 and SEQ ID NO:3;

25 f) a monoclonal antibody that binds to an epitope capable of binding the monoclonal antibody produced by the hybridoma cell line 5.9 or 12.12;

g) a monoclonal antibody that binds to an epitope comprising residues 82-87 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12;

h) a monoclonal antibody that binds to an epitope comprising residues 82-89 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12;

30 i) a monoclonal antibody that competes with the monoclonal antibody 5.9 or 12.12 in a competitive binding assay; and,

j) a monoclonal antibody that is an antigen-binding fragment of a monoclonal antibody of a)-i), wherein said fragment retains the capability of specifically binding to said human CD40 antigen;
said antibody or fragment thereof being free of significant agonistic activity, and whereby
5 when said antibody or fragment thereof binds to said CD40 antigen on said B cell, the growth or differentiation of said B cell is inhibited.

26. The method of embodiment 25, wherein said monoclonal antibody binds to said human CD40 antigen with an affinity (K_D) of at least about 10^{-6} M to about 10^{-12}
10 M.

27. The method of embodiment 25, wherein said fragment is selected from the group consisting of a Fab fragment, an $F(ab')_2$ fragment, an Fv fragment, and a single-chain Fv fragment.
15

28. The method of embodiment 25, wherein said autoimmune disease is selected from the group consisting of systemic lupus erythematosus, autoimmune thrombocytopenic purpura, Rheumatoid arthritis, multiple sclerosis, ankylosing spondylitis, myasthenia gravis, and pemphigus vulgaris.
20

29. A method for treating a cancer characterized by expression of CD40, comprising administering to a human patient an effective amount of a human anti-CD40 monoclonal antibody selected from the group consisting of:

- a) the monoclonal antibody 5.9 or 12.12;
- 25 b) the monoclonal antibody produced by the hybridoma cell line 5.9 or 12.12;
- c) a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:6, the sequence shown in SEQ ID NO:7, the sequence shown in SEQ ID NO:8, both the sequence shown in SEQ
30 ID NO:6 and SEQ ID NO:7, and both the sequence shown in SEQ ID NO:6 and SEQ ID NO:8;

d) a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:2, the sequence shown in SEQ ID NO:4, the sequence shown in SEQ ID NO:5, both the sequence shown in SEQ ID NO:2 and SEQ ID NO:4, and both the sequence shown in SEQ ID NO:2 and SEQ ID NO:5;

e) a monoclonal antibody having an amino acid sequence encoded by a nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of the sequence shown in SEQ ID NO:1, the sequence shown in SEQ ID NO:3, and both the sequence shown in SEQ ID NO:1 and SEQ ID NO:3;

f) a monoclonal antibody that binds to an epitope capable of binding the monoclonal antibody produced by the hybridoma cell line 5.9 or 12.12;

g) a monoclonal antibody that binds to an epitope comprising residues 82-87 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12;

h) a monoclonal antibody that binds to an epitope comprising residues 82-89 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12;

i) a monoclonal antibody that competes with the monoclonal antibody 5.9 or 12.12 in a competitive binding assay; and,

j) a monoclonal antibody that is an antigen-binding fragment of a monoclonal antibody of a)-i), wherein said fragment retains the capability of specifically binding to said human CD40 antigen;
said antibody or fragment thereof being free of significant agonistic activity, and whereby when said antibody or fragment thereof binds to said CD40 antigen on said B cell, the growth or differentiation of said B cell is inhibited.

30. The method of embodiment 29, wherein said monoclonal antibody binds to said human CD40 antigen with an affinity (K_D) of at least about 10^{-6} M to about 10^{-12} M.

31. The method of embodiment 29, wherein said fragment is selected from the group consisting of a Fab fragment, an $F(ab')_2$ fragment, an Fv fragment, and a single-chain Fv fragment.

32. A method for identifying an antibody that has antagonistic activity toward CD40-expressing cells, comprising performing a competitive binding assay with a monoclonal antibody selected from the group consisting of:

- 5 a) the monoclonal antibody 5.9 or 12.12;
- b) the monoclonal antibody produced by the hybridoma cell line 5.9 or 12.12;
- c) a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:6, the sequence shown in
10 SEQ ID NO:7, the sequence shown in SEQ ID NO:8, both the sequence shown in SEQ ID NO:6 and SEQ ID NO:7, and both the sequence shown in SEQ ID NO:6 and SEQ ID NO:8;
- d) a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:2, the sequence shown in
15 SEQ ID NO:4, the sequence shown in SEQ ID NO:5, both the sequence shown in SEQ ID NO:2 and SEQ ID NO:4, and both the sequence shown in SEQ ID NO:2 and SEQ ID NO:5;
- e) a monoclonal antibody having an amino acid sequence encoded by a nucleic acid molecule comprising a nucleotide sequence selected from the group
20 consisting of the sequence shown in SEQ ID NO:1, the sequence shown in SEQ ID NO:3, and both the sequence shown in SEQ ID NO:1 and SEQ ID NO:3;
- f) a monoclonal antibody that binds to an epitope capable of binding the monoclonal antibody produced by the hybridoma cell line 5.9 or 12.12;
- g) a monoclonal antibody that binds to an epitope comprising residues 82-87
25 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12;
- h) a monoclonal antibody that binds to an epitope comprising residues 82-89 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12; and
- i) a monoclonal antibody that is an antigen-binding fragment of a monoclonal antibody of a)-h), wherein said fragment retains the capability of specifically
30 binding to said human CD40 antigen.

33. An antagonist anti-CD40 monoclonal antibody that specifically binds Domain 2 of CD40.

34. The monoclonal antibody of claim 33, wherein said antibody is a human antibody.

35. The monoclonal antibody of claim 34, wherein said antibody is free of significant agonist activity.

36. The monoclonal antibody of claim 33, wherein said antibody has the binding specificity of an antibody selected from the group consisting of the antibody produced by hybridoma cell line 5.9 and the antibody produced by hybridoma cell line 12.12.

37. The monoclonal antibody of claim 33, wherein said antibody is selected from the group consisting of the antibody produced by hybridoma cell line deposited with the ATCC as Patent Deposit No. PTA -5542 and hybridoma cell line deposited with the ATCC as Patent Deposit No. PTA-5543.

38. The monoclonal antibody of claim 33, wherein said antibody has the binding specificity of monoclonal antibody 12.12 or 5.9.

39. The monoclonal antibody of claim 33, wherein said antibody binds to an epitope comprising residues 82-87 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12.

40. The monoclonal antibody of claim 33, wherein said antibody is selected from the group consisting of:

a) a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:2, the sequence shown in SEQ ID NO:4, the sequence shown in SEQ ID NO:5, both the sequence shown in SEQ

ID NO:2 and SEQ ID NO:4, and both the sequence shown in SEQ ID NO:2 and SEQ ID NO:5;

b) a monoclonal antibody having an amino acid sequence encoded by a nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of the sequence shown in SEQ ID NO:1, the sequence shown in SEQ ID NO:3, and both the sequence shown in SEQ ID NO:1 and SEQ ID NO:3;

c) a monoclonal antibody that binds to an epitope capable of binding the monoclonal antibody produced by the hybridoma cell line 12.12;

d) a monoclonal antibody that binds to an epitope comprising residues 82-87 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12;

e) a monoclonal antibody that competes with the monoclonal antibody 12.12 in a competitive binding assay; and

f) a monoclonal antibody that is an antigen-binding fragment of the 12.12 monoclonal antibody or the foregoing monoclonal antibodies in preceding items (a)-(e), where the fragment retains the capability of specifically binding to the human CD40 antigen.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows binding of 5.9 and 12.12 monoclonal antibodies to CD40 on the surface of lymphoma cell line (Ramos).

Figures 2A and 2B illustrate binding properties of the 5.9 and 12.12 monoclonal anti-CD40 antibodies relative to CD40 ligand. Figure 2A shows that binding of 5.9 and 12.12 monoclonal antibodies to cell surface CD40 prevents subsequent CD40-ligand binding. Figure 2B shows that the 5.9 and 12.12 monoclonal antibodies can compete off CD40 ligand pre-bound to cell surface CD40.

Figures 3A and 3B show ADCC activity of the candidate monoclonal antibodies 5.9 and 12.12 against cancer cells from the lymph nodes of non-Hodgkin's lymphoma (NHL) patients. Enriched NK cells from a normal volunteer donor either fresh after isolation (Figure 3A) or after culturing overnight at 37°C (Figure 3B) were used as effector cells in this assay. As NHL cells also express CD20, the target antigen for

rituximab (Rituxan®), ADCC activity of the candidate mAbs was compared with that of rituximab.

Figure 4 demonstrates *in vivo* anti-tumor activity of monoclonal antibodies 5.9 and 12.12 compared to that of rituximab using an unstaged nude mouse xenograft B cell lymphoma (Namalwa) model.

Figure 5 demonstrates *in vivo* anti-tumor activity of monoclonal antibodies 5.9 and 12.12 compared to that of rituximab using an unstaged nude mouse xenograft B cell lymphoma (Daudi) model. RC, resistance to tumor challenge.

Figure 6 demonstrates *in vivo* anti-tumor activity of monoclonal antibodies 5.9 and 12.12 compared to that of rituximab using a staged nude mouse xenograft B cell lymphoma (Daudi) model. CR, complete regression.

Figure 7 shows the protocol used for determining the number of CD20 and CD40 molecules on Namalwa and Daudi cells.

Figure 8 shows comparative ADCC of the mAb 12.12 and rituximab against Daudi lymphoma cells.

Figure 9 sets forth the amino acid sequences for the light and heavy chains of the mAb 12.12. The leader (residues 1-20 of SEQ ID NO:2), variable (residues 21-132 of SEQ ID NO:2), and constant (residues 133-239 of SEQ ID NO:2) regions of the light chain are shown in Figure 9A. The leader (residues 1-19 of SEQ ID NO:4), variable (residues 20-139 of SEQ ID NO:4), and constant (residues 140-469 of SEQ ID NO:4) regions of the heavy chain are shown in Figure 9B. The alternative constant region for the heavy chain of the mAb 12.12 shown in Figure 9B reflects a substitution of a serine residue for the alanine residue at position 153 of SEQ ID NO:4. The complete sequence for this variant of the heavy chain of the mAb 12.12 is set forth in SEQ ID NO:5.

Figure 10 sets forth the amino acid sequences for the light and heavy chains of mAb 5.9. The leader (residues 1-20 of SEQ ID NO:6), variable (residues 21-132 of SEQ ID NO:6), and constant (residues 133-239 of SEQ ID NO:6) regions of the light chain are shown in Figure 10A. The leader (residues 1-19 of SEQ ID NO:7), variable (residues 20-144 of SEQ ID NO:7), and constant (residues 145-474 of SEQ ID NO:7) regions of the heavy chain are shown in Figure 10B. The alternative constant region for the heavy chain of the mAb 5.9 shown in Figure 10B reflects a substitution of a serine residue for

"Native antibodies" and "native immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity determining regions (CDRs) or hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework (FR) regions. The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a β -sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β -sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat *et al.* (1991) *NIH Publ. No. 91-3242*, Vol. I, pages 647-669).

The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as Fc receptor binding, participation of the antibody in antibody-dependent cellular toxicity, initiation of complement dependent cytotoxicity, and mast cell degranulation.

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody that are responsible for antigen binding. The hypervariable region

comprises amino acid residues from a "complementarity determining region" or "CDR" (i.e., residues 24-34 (L1), 50-56 (L2), and 89-97 (L3) in the light-chain variable domain and 31-35 (H1), 50-65 (H2), and 95-102 (H3) in the heavy-chain variable domain; Kabat *et al.* (1991) *Sequences of Proteins of Immunological Interest* (5th ed., Public Health Service, National Institute of Health, Bethesda, MD) and/or those residues from a "hypervariable loop" (i.e., residues 26-32(L1), 50-52 (L2), and 91-96 (L3) in the light-chain variable domain and 26-32(H1), 53-55 (H2), and 96-101 (H3) in the heavy-chain variable domain; Clothia and Lesk (1987) *J. Mol. Biol.* 196:901-917). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues.

"Antibody fragments" comprise a portion of an intact antibody, preferably the antigen-binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', F(ab')₂, and Fv fragments; diabodies; linear antibodies (Zapata *et al.* (1995) *Protein Eng.* 8(10):1057-1062); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an F(ab')₂ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment that contains a complete antigen recognition and binding site. In a two-chain Fv species, this region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv species, one heavy- and one light-chain variable domain can be covalently linked by flexible peptide linker such that the light and heavy chains can associate in a "dimeric" structure analogous to that in a two-chain Fv species. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (C_H1) of the heavy chain. Fab fragments differ from Fab' fragments by the addition of a few residues at the carboxy terminus of the heavy-chain C_H1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab')₂ antibody fragments originally were produced as pairs of Fab' fragments that have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called alpha, delta, epsilon, gamma, and mu, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known. Different isotypes have different effector functions. For example, IgG1 and IgG3 isotypes have antibody-dependent cell-mediated cytotoxicity (ADCC) activity.

The word "label" when used herein refers to a detectable compound or composition that is conjugated directly or indirectly to the antibody so as to generate a "labeled" antibody. The label may be detectable by itself (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition that is detectable. Radionuclides that can serve as detectable labels include, for example, I-131, I-123, I-125, Y-90, Re-188, Re-186, At-211, Cu-67, Bi-212, and Pd-109. The label might also be a non-detectable entity such as a toxin.

The term "antagonist" is used in the broadest sense, and includes any molecule that partially or fully blocks, inhibits, or neutralizes a biological activity of a native target disclosed herein or the transcription or translation thereof.

"Carriers" as used herein include pharmaceutically acceptable carriers, excipients, or stabilizers that are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN, polyethylene glycol (PEG), and Pluronic. Administration "in combination with" one or more further therapeutic agents includes simultaneous (concurrent) and consecutive administration in any order.

A "host cell," as used herein, refers to a microorganism or a eukaryotic cell or cell line cultured as a unicellular entity that can be, or has been, used as a recipient for a recombinant vector or other transfer polynucleotides, and include the progeny of the original cell that has been transfected. It is understood that the progeny of a single cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation.

"Human effector cells" are leukocytes that express one or more FcRs and perform effector functions. Preferably, the cells express at least FcγRIII and carry out antigen-dependent cell-mediated cytotoxicity (ADCC) effector function. Examples of human leukocytes that mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, macrophages, eosinophils, and neutrophils, with PBMCs and NK cells being preferred. Antibodies that have ADCC activity are typically of the IgG1 or IgG3 isotype. Note that in addition to isolating IgG1 and IgG3 antibodies, such ADCC-mediating antibodies can be made by engineering a variable region from a

non-ADCC antibody or variable region fragment to an IgG1 or IgG3 isotype constant region.

The terms "Fc receptor" or "FcR" are used to describe a receptor that binds to the Fc region of an antibody. The preferred FcR is a native-sequence human FcR. Moreover, a preferred FcR is one that binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of these receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain (see Daeron (1997) *Annu. Rev. Immunol.* 15:203-234). FcRs are reviewed in Ravetch and Kinet (1991) *Annu. Rev. Immunol.* 9:457-492 (1991); Capel *et al.* (1994) *Immunomethods* 4:25-34; and de Haas *et al.* (1995) *J. Lab. Clin. Med.* 126:330-341. Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer *et al.* (1976) *J. Immunol.* 117:587 and Kim *et al.* (1994) *J. Immunol.* 24:249 (1994)).

There are a number of ways to make human antibodies. For example, secreting cells can be immortalized by infection with the Epstein-Barr virus (EBV). However, EBV-infected cells are difficult to clone and usually produce only relatively low yields of immunoglobulin (James and Bell (1987) *J. Immunol. Methods* 100:5-40). In the future, the immortalization of human B cells might possibly be achieved by introducing a defined combination of transforming genes. Such a possibility is highlighted by a recent demonstration that the expression of the telomerase catalytic subunit together with the SV40 large oncoprotein and an oncogenic allele of H-ras resulted in the tumorigenic conversion of normal human epithelial and fibroblast cells (Hahn *et al.* (1999) *Nature* 400:464-468). It is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a repertoire of human antibodies in the absence of endogenous immunoglobulin production (Jakobovits *et al.* (1993) *Nature*

362:255-258; Lonberg and Huszar (1995) *Int. Rev. Immunol.* 13:65-93; Fishwild *et al.* (1996) *Nat. Biotechnol.* 14:845-851; Mendez *et al.* (1997) *Nat. Genet.* 15:146-156; Green (1999) *J. Immunol. Methods* 231:11-23; Tomizuka *et al.* (2000) *Proc. Natl. Acad. Sci. USA* 97:722-727; reviewed in Little *et al.* (2000) *Immunol. Today* 21:364-370). For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production (Jakobovits *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:2551-2555). Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice results in the production of human antibodies upon antigen challenge (Jakobovits *et al.* (1993) *Nature* 362:255-258). Mendez *et al.* (1997) (*Nature Genetics* 15:146-156) have generated a line of transgenic mice that, when challenged with an antigen, generates high affinity fully human antibodies. This was achieved by germ-line integration of megabase human heavy-chain and light-chain loci into mice with deletion into endogenous J_H segment as described above. The XenoMouse II harbors 1,020 kb of human heavy-chain locus containing approximately 66 V_H genes, complete D_H and J_H regions, and three different constant regions, and also harbors 800 kb of human κ locus containing 32 V κ genes, J κ segments, and C κ genes. The antibodies produced in these mice closely resemble that seen in humans in all respects, including gene rearrangement, assembly, and repertoire. The human antibodies are preferentially expressed over endogenous antibodies due to deletion in endogenous segment that prevents gene rearrangement in the murine locus. Such mice may be immunized with an antigen of particular interest.

Sera from such immunized animals may be screened for antibody reactivity against the initial antigen. Lymphocytes may be isolated from lymph nodes or spleen cells and may further be selected for B cells by selecting for CD138-negative and CD19-positive cells. In one aspect, such B cell cultures (BCCs) may be fused to myeloma cells to generate hybridomas as detailed above.

In another aspect, such B cell cultures may be screened further for reactivity against the initial antigen, preferably. Such screening includes ELISA with the target/antigen protein, a competition assay with known antibodies that bind the antigen of

interest, and in vitro binding to transiently transfected CHO or other cells that express the target antigen.

The present invention is directed to compositions and methods for treating human patients with diseases mediated by stimulation of CD40 signaling on CD40-expressing cells. The methods involve treatment with an anti-CD40 antibody of the invention, or an antigen-binding fragment thereof, where administration of the antibody or antigen-binding fragment thereof promotes a positive therapeutic response within the patient undergoing this method of therapy. Anti-CD40 antibodies suitable for use in the methods of the invention specifically bind a human CD40 antigen expressed on the surface of a human cell and are free of significant agonist activity, but exhibit antagonist activity when bound to the CD40 antigen on a human CD40-expressing cell. These anti-CD40 antibodies and antigen-binding fragments thereof are referred to herein as antagonist anti-CD40 antibodies. Such antibodies include, but are not limited to, the fully human monoclonal antibodies 5.9 and 12.12 described below and monoclonal antibodies having the binding characteristics of monoclonal antibodies 5.9 and 12.12.

Antibodies that have the binding characteristics of monoclonal antibodies 5.9 and 12.12 include antibodies that competitively interfere with binding CD40 and/or bind the same epitopes as 5.9 and 12.12. One of skill could determine whether an antibody competitively interferes with 5.9 or 12.12 using standard methods.

When these antibodies bind CD40 displayed on the surface of human cells, such as human B cells, the antibodies are free of significant agonist activity; in some embodiments, their binding to CD40 displayed on the surface of human cells results in inhibition of proliferation and differentiation of these human cells. Thus, the antagonist anti-CD40 antibodies suitable for use in the methods of the invention include those monoclonal antibodies that can exhibit antagonist activity toward normal and malignant human cells expressing the cell-surface CD40 antigen.

In some embodiments, the anti-CD40 antibodies of the invention exhibit increased anti-tumor activity relative to the chimeric anti-CD20 monoclonal antibody IDEC-C2B8, where anti-tumor activity is assayed with equivalent amounts of these antibodies in a nude mouse xenograft tumor model using human lymphoma cell lines. IDEC-C2B8 (IDEC Pharmaceuticals Corp., San Diego, California; commercially

available under the tradename Rituxan®, also referred to as rituximab) is a chimeric anti-CD20 monoclonal antibody containing human IgG1 and kappa constant regions with murine variable regions isolated from a murine anti-CD20 monoclonal antibody, IDEC-2B8 (Reff *et al.* (1994) *Blood* 83:435-445). Rituximab® is licensed for treatment of relapsed B cell low-grade or follicular non-Hodgkin's lymphoma (NHL). The discovery of antibodies with superior anti-tumor activity compared to Rituximab® could drastically improve methods of cancer therapy for B cell lymphomas, particularly B cell non-Hodgkin's lymphoma.

Suitable nude mouse xenograft tumor models include those using the human Burkitt's lymphoma cell lines known as Namalwa and Daudi. Preferred embodiments assay anti-tumor activity in a staged nude mouse xenograft tumor model using the Daudi human lymphoma cell line as described herein below in Example 17. A staged nude mouse xenograft tumor model using the Daudi lymphoma cell line is more effective at distinguishing the therapeutic efficacy of a given antibody than is an unstaged model, as in the staged model antibody dosing is initiated only after the tumor has reached a measurable size. In the unstaged model, antibody dosing is initiated generally within about 1 day of tumor inoculation and before a palpable tumor is present. The ability of an antibody to outperform Rituxan® (i.e., to exhibit increased anti-tumor activity) in a staged model is a strong indication that the antibody will be more therapeutically effective than Rituxan®. Moreover, in the Daudi model, anti-CD20, the target for Rituxan® is expressed on the cell surface at a higher level than is CD40.

By "equivalent amount" of the anti-CD40 antibody of the invention and Rituxan® is intended the same mg dose is administered on a per weight basis. Thus, where the anti-CD40 antibody of the invention is dosed at 0.01 mg/kg body weight of the mouse used in the tumor model, Rituxan® is also dosed at 0.01 mg/kg body weight of the mouse. Similarly, where the anti-CD40 antibody of the invention is dosed at 0.1, 1, or 10 mg/kg body weight of the mouse used in the tumor model, the Rituxan® is also dosed at 0.1, 1, or 10 mg/kg, respectively, of the body weight of the mouse.

When administered in the nude mouse xenograft tumor model, some antibodies of the invention result in significantly less tumor volume than an equivalent amount of Rituxan®. Thus, for example, the fully human monoclonal antibody 12.12 exhibits at

least a 20% increase in anti-tumor activity relative to that observed with an equivalent dose of Rituxan when assayed in the staged nude mouse xenograft tumor model using the Daudi human lymphoma cell line in the manner described in Examples herein below, and can exhibit as much as a 50% to 60% increase in anti-tumor activity in this assay. This increased anti-tumor activity is reflected in the greater reduction in tumor volume observed with the anti-CD40 antibody of the invention when compared to the equivalent dose of Rituxan®. Thus, for example, depending upon the length of time after tumor inoculation, the monoclonal antibody 12.12 can exhibit a tumor volume that is about one-third to about one-half that observed for an equivalent dose of Rituxan®.

Another difference in antibody efficacy is to measure *in vitro* the concentration of antibody needed to obtain the maximum lysis of tumor cells *in vitro* in the presence of NK cells. For example, the anti-CD40 antibodies of the invention reach maximum lysis of Daudi cells at an EC50 of less than 1/2, and preferably 1/4, and most preferably, 1/10 the concentration of Rituxan®.

In addition to the monoclonal antibody 12.12, other anti-CD40 antibodies that would share the characteristics of having significantly greater efficacy than equivalent amounts of Rituxan® in the assays described above include, but are not limited to: (1) the monoclonal antibody produced by the hybridoma cell line 12.12; (2) a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence in SEQ ID NO:2, the sequence in SEQ ID NO:4, the sequence in SEQ ID NO:5, both the sequence in SEQ ID NO:2 and SEQ ID NO:4, and both the sequence in SEQ ID NO:2 and SEQ ID NO:5; (3) a monoclonal antibody having an amino acid sequence encoded by a nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of the nucleotide sequence in SEQ ID NO:1, the nucleotide sequence in SEQ ID NO:3, and both the sequence in SEQ ID NO:1 and SEQ ID NO:3; (4) a monoclonal antibody that binds to an epitope capable of binding the monoclonal antibody produced by the hybridoma cell line 12.12; (5) a monoclonal antibody that binds to an epitope comprising residues 82-87 of the amino acid sequence in SEQ ID NO:10 or SEQ ID NO:12; (6) a monoclonal antibody that competes with the monoclonal antibody 12.12 in a competitive binding assay; and (7) a monoclonal antibody that is an antigen-binding fragment of the 12.12 monoclonal antibody or the foregoing monoclonal

antibodies in preceding items (1)-(6), where the fragment retains the capability of specifically binding to the human CD40 antigen.

Antagonist Anti-CD40 Antibodies

5 The monoclonal antibodies 5.9 and 12.12 represent suitable antagonist anti-CD40 antibodies for use in the methods of the present invention. The 5.9 and 12.2 antibodies are fully human anti-CD40 monoclonal antibodies of the IgG₁ isotype produced from the hybridoma cell lines 131.2F8.5.9 (referred to herein as the cell line 5.9) and 153.8E2.D10.D6.12.12 (referred to herein as the cell line 12.12). These cell lines were
10 created using splenocytes from immunized xenotypic mice containing the human IgG₁ heavy chain locus and the human K chain locus (Abgenix). The spleen cells were fused with the mouse myeloma SP2/0 cells (Sierra BioSource). The resulting hybridomas were sub-cloned several times to create the stable monoclonal cell lines 5.9 and 12.12. Other antibodies of the invention may be prepared similarly using mice transgenic for human
15 immunoglobulin loci or by other methods known in the art and/or described herein.

 The nucleotide and amino acid sequences of the variable regions of the 12.12 antibody, and the amino acid sequences of the variable regions of the 5.9 antibody, are disclosed. More particularly, the amino acid sequences for the leader, variable, and constant regions for the light chain and heavy chain for mAb 12.12 are set forth in
20 Figures 9A and 9B, respectively. See also SEQ ID NO:2 (complete sequence for the light chain of mAb 12.12), SEQ ID NO:4 (complete sequence for the heavy chain for mAb 12.12), and SEQ ID NO:5 (complete sequence for a variant of the heavy chain for mAb 12.12 set forth in SEQ ID NO:4, where the variant comprises a serine substitution for the alanine residue at position 153 of SEQ ID NO:4). The nucleotide sequences
25 encoding the light chain and heavy chain for mAb 12.12 are set forth in Figures 11A and 11B, respectively. See also SEQ ID NO:1 (coding sequence for the light chain for mAb 12.12), and SEQ ID NO:3 (coding sequence for the heavy chain for mAb 12.12). The amino acid sequences for the leader, variable, and constant regions for the light chain and heavy chain of the 5.9 mAb are set forth in Figures 10A and 10B, respectively. See also
30 SEQ ID NO:6 (complete sequence for the light chain of mAb 5.9), SEQ ID NO:7 (complete sequence for the heavy chain of mAb 5.9), and SEQ ID NO:8 (complete

sequence for a variant of the heavy chain of mAb 5.9 set forth in SEQ ID NO:7, where the variant comprises a serine substitution for the alanine residue at position 158 of SEQ ID NO:7). Further, hybridomas expressing 5.9 and 12.12 antibodies have been deposited with the ATCC with a patent deposit designation of PTA-5542 and PTA-5543, respectively.

In addition to antagonist activity, it is preferable that anti-CD40 antibodies of this invention have another mechanism of action against a tumor cell. For example, native 5.9 and 12.12 antibodies have ADCC activity. Alternatively, the variable regions of the 5.9 and 12.12 antibodies can be expressed on another antibody isotype that has ADCC activity. It is also possible to conjugate native forms, recombinant forms, or antigen-binding fragments of 5.9 or 12.12 to a cytotoxic toxin.

The 5.9 and 12.12 monoclonal antibodies bind soluble CD40 in ELISA-type assays, prevent the binding of CD40-ligand to cell-surface CD40, and displace the pre-bound CD40-ligand, as determined by flow cytometric assays. Antibodies 5.9 and 12.12 compete with each other for binding to CD40 but not with 15B8, the anti-CD40 monoclonal antibody described in U.S. Provisional Application Serial No. 60/237,556, titled "*Human Anti-CD40 Antibodies*," filed October 2, 2000, and PCT International Application No. PCT/US01/30857, also titled "*Human Anti-CD40 Antibodies*," filed October 2, 2001 (Attorney Docket No. PP16092.003), both of which are herein incorporated by reference in their entirety. When tested *in vitro* for effects on proliferation of B cells from normal human subjects, 5.9 and 12.12 act as antagonistic anti-CD40 antibodies. Furthermore, 5.9 and 12.12 do not induce strong proliferation of human lymphocytes from normal subjects. These antibodies are able to kill CD40-expressing target cells by antibody dependent cellular cytotoxicity (ADCC). The binding affinity of 5.9 for human CD40 is 1.2×10^{-8} M and the binding affinity of 12.12 is 5×10^{-10} M, as determined by the Biacore™ assay.

Suitable antagonist anti-CD40 antibodies for use in the methods of the present invention exhibit a strong single-site binding affinity for the CD40 cell-surface antigen. The monoclonal antibodies of the invention exhibit a dissociation equilibrium constant (K_D) for CD40 of at least 10^{-5} M, at least 3×10^{-5} M, preferably at least 10^{-6} M to 10^{-7} M, more preferably at least 10^{-8} M to about 10^{-12} M, measured using a standard assay such as

Biacore™. Biacore analysis is known in the art and details are provided in the "BIAapplications handbook." Methods described in WO 01/27160 can be used to modulate the binding affinity.

By "CD40 antigen," "CD40 cell surface antigen," "CD40 receptor," or "CD40" is intended a transmembrane glycoprotein that belongs to the tumor necrosis factor (TNF) receptor family (see, for example, U.S. Patent Nos. 5,674,492 and 4,708,871; Stamenkovic *et al.* (1989) *EMBO* 8:1403; Clark (1990) *Tissue Antigens* 36:33; Barclay *et al.* (1997) *The Leucocyte Antigen Facts Book* (2d ed.; Academic Press, San Diego)).

Two isoforms of human CD40, encoded by alternatively spliced transcript variants of this gene, have been identified. The first isoform (also known as the "long isoform" or

"isoform 1") is expressed as a 277-amino-acid precursor polypeptide (SEQ ID NO:12 (first reported as GenBank Accession No. CAA43045, and identified as isoform 1 in GenBank Accession No. NP_001241), encoded by SEQ ID NO:11 (see GenBank

Accession Nos. X60592 and NM_001250)), which has a signal sequence represented by

the first 19 residues. The second isoform (also known as the "short isoform" or "isoform 2") is expressed as a 203-amino-acid precursor polypeptide (SEQ ID NO:10 (GenBank Accession No. NP_690593), encoded by SEQ ID NO:9 (GenBank Accession No.

NM_152854)), which also has a signal sequence represented by the first 19 residues. The precursor polypeptides of these two isoforms of human CD40 share in common their first

165 residues (i.e., residues 1-165 of SEQ ID NO:10 and SEQ ID NO:12). The precursor polypeptide of the short isoform (shown in SEQ ID NO:10) is encoded by a transcript variant (SEQ ID NO:9) that lacks a coding segment, which leads to a translation frame shift; the resulting CD40 isoform contains a shorter and distinct C-terminus (residues

166-203 of SEQ ID NO:10) from that contained in the long isoform of CD40 (C-terminus

shown in residues 166-277 of SEQ ID NO:12). For purposes of the present invention, the term "CD40 antigen," "CD40 cell surface antigen," "CD40 receptor," or "CD40"

encompasses both the short and long isoforms of CD40. The anti-CD40 antibodies of the present invention bind to an epitope of human CD40 that resides at the same location within either the short isoform or long isoform of this cell surface antigen as noted herein

below.

The CD40 antigen is displayed on the surface of a variety of cell types, as described elsewhere herein. By "displayed on the surface" and "expressed on the surface" is intended that all or a portion of the CD40 antigen is exposed to the exterior of the cell. The displayed or expressed CD40 antigen may be fully or partially glycosylated.

5 By "agonist activity" is intended that the substance functions as an agonist. An agonist combines with a receptor on a cell and initiates a reaction or activity that is similar to or the same as that initiated by the receptor's natural ligand. An agonist of CD40 induces any or all of, but not limited to, the following responses: B cell proliferation and differentiation, antibody production, intercellular adhesion, B cell
10 memory generation, isotype switching, up-regulation of cell-surface expression of MHC Class II and CD80/86, and secretion of pro-inflammatory cytokines such as IL-8, IL-12, and TNF. By "antagonist activity" is intended that the substance functions as an antagonist. An antagonist of CD40 prevents or reduces induction of any of the responses induced by binding of the CD40 receptor to an agonist ligand, particularly CD40L. The
15 antagonist may reduce induction of any one or more of the responses to agonist binding by 5%, 10%, 15%, 20%, 25%, 30%, 35%, preferably 40%, 45%, 50%, 55%, 60%, more preferably 70%, 80%, 85%, and most preferably 90%, 95%, 99%, or 100%. Methods for measuring anti-CD40 antibody and CD40-ligand binding specificity and antagonist activity are known to one of skill in the art and include, but are not limited to, standard
20 competitive binding assays, assays for monitoring immunoglobulin secretion by B cells, B cell proliferation assays, Banchereau-Like-B cell proliferation assays, T cell helper assays for antibody production, co-stimulation of B cell proliferation assays, and assays for up-regulation of B cell activation markers. See, for example, such assays disclosed in WO 00/75348 and U.S. Patent No. 6,087,329, herein incorporated by reference.

25 By "significant" agonist activity is intended an agonist activity of at least 30%, 35%, 40%, 45%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% greater than the agonist activity induced by a neutral substance or negative control as measured in an assay of a B cell response. Preferably, "significant" agonist activity is an agonist activity that is at least 2-fold greater or at least 3-fold greater than the agonist activity induced by
30 a neutral substance or negative control as measured in an assay of a B cell response. Thus, for example, where the B cell response of interest is B cell proliferation,

"significant" agonist activity would be induction of a level of B cell proliferation that is at least 2-fold greater or at least 3-fold greater than the level of B cell proliferation induced by a neutral substance or negative control. In one embodiment, a non-specific immunoglobulin, for example IgG1, that does not bind to CD40 serves as the negative control. A substance "free of significant agonist activity" would exhibit an agonist activity of not more than about 25% greater than the agonist activity induced by a neutral substance or negative control, preferably not more than about 20% greater, 15% greater, 10% greater, 5% greater, 1% greater, 0.5% greater, or even not more than about 0.1% greater than the agonist activity induced by a neutral substance or negative control as measured in an assay of a B cell response. The antagonist anti-CD40 antibodies useful in the methods of the present invention are free of significant agonist activity as noted above when bound to a CD40 antigen on a human cell. In one embodiment of the invention, the antagonist anti-CD40 antibody is free of significant agonist activity in one B cell response. In another embodiment of the invention, the antagonist anti-CD40 antibody is free of significant agonist activity in assays of more than one B cell response (e.g., proliferation and differentiation, or proliferation, differentiation, and antibody production).

As used herein "anti-CD40 antibody" encompasses any antibody that specifically recognizes the CD40 B cell surface antigen, including polyclonal antibodies, monoclonal antibodies, single-chain antibodies, and fragments thereof such as Fab, F(ab')₂, F_v, and other fragments which retain the antigen binding function of the parent anti-CD40 antibody. Of particular interest to the methods of the present invention are the binding properties exhibited by the 5.9 and 12.12 monoclonal antibodies described above.

Polyclonal sera may be prepared by conventional methods. In general, a solution containing the CD40 antigen is first used to immunize a suitable animal, preferably a mouse, rat, rabbit, or goat. Rabbits or goats are preferred for the preparation of polyclonal sera due to the volume of serum obtainable, and the availability of labeled anti-rabbit and anti-goat antibodies.

Polyclonal sera can be prepared in a transgenic animal, preferably a mouse bearing human immunoglobulin loci. In a preferred embodiment, Sf9 cells expressing CD40 are used as the immunogen. Immunization can also be performed by mixing or

emulsifying the antigen-containing solution in saline, preferably in an adjuvant such as Freund's complete adjuvant, and injecting the mixture or emulsion parenterally (generally subcutaneously or intramuscularly). A dose of 50-200 µg/injection is typically sufficient. Immunization is generally boosted 2-6 weeks later with one or more injections of the protein in saline, preferably using Freund's incomplete adjuvant. One may alternatively generate antibodies by *in vitro* immunization using methods known in the art, which for the purposes of this invention is considered equivalent to *in vivo* immunization. Polyclonal antisera are obtained by bleeding the immunized animal into a glass or plastic container, incubating the blood at 25°C for one hour, followed by incubating at 4°C for 2-18 hours. The serum is recovered by centrifugation (e.g., 1,000 x g for 10 minutes). About 20-50 ml per bleed may be obtained from rabbits.

Production of the Sf 9 (*Spodoptera frugiperda*) cells is disclosed in U.S. Patent No. 6,004,552, incorporated herein by reference. Briefly, sequences encoding human CD40 were recombined into a baculovirus using transfer vectors. The plasmids were co-transfected with wild-type baculovirus DNA into Sf 9 cells. Recombinant baculovirus-infected Sf 9 cells were identified and clonally purified.

Preferably the antibody is monoclonal in nature. By "monoclonal antibody" is intended an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. The term is not limited regarding the species or source of the antibody. The term encompasses whole immunoglobulins as well as fragments such as Fab, F(ab')₂, Fv, and others which retain the antigen binding function of the antibody. Monoclonal antibodies are highly specific, being directed against a single antigenic site, i.e., the CD40 cell surface antigen in the present invention. Furthermore, in contrast to conventional (polyclonal) antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance

with the present invention may be made by the hybridoma method first described by Kohler *et al.* (1975) *Nature* 256:495, or may be made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in, for example, Clackson *et al.* (1991) *Nature* 352:624-628; Marks *et al.* (1991) *J. Mol. Biol.* 222:581-597; and U.S. Patent No. 5,514,548.

By "epitope" is intended the part of an antigenic molecule to which an antibody is produced and to which the antibody will bind. Epitopes can comprise linear amino acid residues (*i.e.*, residues within the epitope are arranged sequentially one after another in a linear fashion), nonlinear amino acid residues (referred to herein as "nonlinear epitopes"; these epitopes are not arranged sequentially), or both linear and nonlinear amino acid residues.

Monoclonal antibodies can be prepared using the method of Kohler *et al.* (1975) *Nature* 256:495-496, or a modification thereof. Typically, a mouse is immunized with a solution containing an antigen. Immunization can be performed by mixing or emulsifying the antigen-containing solution in saline, preferably in an adjuvant such as Freund's complete adjuvant, and injecting the mixture or emulsion parenterally. Any method of immunization known in the art may be used to obtain the monoclonal antibodies of the invention. After immunization of the animal, the spleen (and optionally, several large lymph nodes) are removed and dissociated into single cells. The spleen cells may be screened by applying a cell suspension to a plate or well coated with the antigen of interest. The B cells expressing membrane bound immunoglobulin specific for the antigen bind to the plate and are not rinsed away. Resulting B cells, or all dissociated spleen cells, are then induced to fuse with myeloma cells to form hybridomas, and are cultured in a selective medium. The resulting cells are plated by serial dilution and are assayed for the production of antibodies that specifically bind the antigen of interest (and that do not bind to unrelated antigens). The selected monoclonal antibody (mAb)-secreting hybridomas are then cultured either *in vitro* (*e.g.*, in tissue culture bottles or hollow fiber reactors), or *in vivo* (as ascites in mice).

As an alternative to the use of hybridomas, antibody can be produced in a cell line such as a CHO cell line, as disclosed in U.S. Patent Nos. 5,545,403; 5,545,405; and

5,998,144; incorporated herein by reference. Briefly the cell line is transfected with vectors capable of expressing a light chain and a heavy chain, respectively. By transfecting the two proteins on separate vectors, chimeric antibodies can be produced. Another advantage is the correct glycosylation of the antibody.

5 Monoclonal antibodies to CD40 are known in the art. See, for example, the sections dedicated to B-cell antigen in McMichael, ed. (1987; 1989) *Leukocyte Typing III and IV* (Oxford University Press, New York); U.S. Patent Nos. 5,674,492; 5,874,082; 5,677,165; 6,056,959; WO 00/63395; International Publication Nos. WO 02/28905 and WO 02/28904; Gordon *et al.* (1988) *J. Immunol.* 140:1425; Valle *et al.* (1989) *Eur. J. Immunol.* 19:1463; Clark *et al.* (1986) *PNAS* 83:4494; Paulie *et al.* (1989) *J. Immunol.* 142:590; Gordon *et al.* (1987) *Eur. J. Immunol.* 17:1535; Jabara *et al.* (1990) *J. Exp. Med.* 172:1861; Zhang *et al.* (1991) *J. Immunol.* 146:1836; Gascan *et al.* (1991) *J. Immunol.* 147:8; Banchereau *et al.* (1991) *Clin. Immunol. Spectrum* 3:8; and Banchereau *et al.* (1991) *Science* 251:70; all of which are herein incorporated by reference. Of particular interest to the present invention are the antagonist anti-CD40 antibodies disclosed herein that share the binding characteristics of the monoclonal antibodies 5.9 and 12.12 described above.

The term "CD40-antigen epitope" as used herein refers to a molecule that is capable of immunoreactivity with the anti-CD40 monoclonal antibodies of this invention, excluding the CD40 antigen itself. CD40-antigen epitopes may comprise proteins, protein fragments, peptides, carbohydrates, lipids, and other molecules, but for the purposes of the present invention are most commonly proteins, short oligopeptides, oligopeptide mimics (i.e., organic compounds which mimic the antibody binding properties of the CD40 antigen), or combinations thereof. Suitable oligopeptide mimics are described, inter alia, in PCT application US 91/04282.

Additionally, the term "anti-CD40 antibody" as used herein encompasses chimeric anti-CD40 antibodies; such chimeric anti-CD40 antibodies for use in the methods of the invention have the binding characteristics of the 5.9 and 12.12 monoclonal antibodies described herein. By "chimeric" antibodies is intended antibodies that are most preferably derived using recombinant deoxyribonucleic acid techniques and which comprise both human (including immunologically "related" species, e.g.,

chimpanzee) and non-human components. Thus, the constant region of the chimeric antibody is most preferably substantially identical to the constant region of a natural human antibody; the variable region of the chimeric antibody is most preferably derived from a non-human source and has the desired antigenic specificity to the CD40 cell-surface antigen. The non-human source can be any vertebrate source that can be used to generate antibodies to a human CD40 cell-surface antigen or material comprising a human CD40 cell-surface antigen. Such non-human sources include, but are not limited to, rodents (e.g., rabbit, rat, mouse, etc.; see, for example, U.S. Patent No. 4,816,567, herein incorporated by reference) and non-human primates (e.g., Old World Monkey, Ape, etc.; see, for example, U.S. Patent Nos. 5,750,105 and 5,756,096; herein incorporated by reference). As used herein, the phrase "immunologically active" when used in reference to chimeric anti-CD40 antibodies means a chimeric antibody that binds human CD40.

Chimeric and humanized anti-CD40 antibodies are also encompassed by the term anti-CD40 antibody as used herein. Chimeric antibodies comprise segments of antibodies derived from different species. Rituxan® is an example of a chimeric antibody with a murine variable region and a human constant region.

By "humanized" is intended forms of anti-CD40 antibodies that contain minimal sequence derived from non-human immunoglobulin sequences. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region (also known as complementarity determining region or CDR) of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit, or nonhuman primate having the desired specificity, affinity, and capacity. The phrase "complementarity determining region" refers to amino acid sequences which together define the binding affinity and specificity of the natural Fv region of a native immunoglobulin binding site. See, e.g., Chothia *et al* (1987) *J. Mol. Biol.* 196:901-917; Kabat *et al* (1991) U. S. Dept. of Health and Human Services, NIH Publication No. 91-3242). The phrase "constant region" refers to the portion of the antibody molecule that confers effector functions. In previous work directed towards producing non-immunogenic antibodies for use in therapy of human disease, mouse constant regions were substituted by human constant regions. The

constant regions of the subject humanized antibodies were derived from human immunoglobulins. However, these humanized antibodies still elicited an unwanted and potentially dangerous immune response in humans and there was a loss of affinity. Humanized anti-CD40 antibodies for use in the methods of the present invention have binding characteristics similar to those exhibited by the 5.9 and 12.12 monoclonal antibodies described herein.

Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al.* (1986) *Nature* 321:522-525; Riechmann *et al.* (1988) *Nature* 332:323-327; Verhoeyen *et al.* (1988) *Science* 239:1534-1536), by substituting rodent or mutant rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. See also U.S. Patent Nos. 5,225,539; 5,585,089; 5,693,761; 5,693,762; 5,859,205; herein incorporated by reference. In some instances, residues within the framework regions of one or more variable regions of the human immunoglobulin are replaced by corresponding non-human residues (see, for example, U.S. Patent Nos. 5,585,089; 5,693,761; 5,693,762; and 6,180,370). Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance (e.g., to obtain desired affinity). In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable regions correspond to those of a non-human immunoglobulin and all or substantially all of the framework regions are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details see Jones *et al.* (1986) *Nature* 331:522-525; Riechmann *et al.* (1988) *Nature* 332:323-329; and Presta (1992) *Curr. Op. Struct. Biol.* 2:593-596; herein incorporated by reference. Accordingly, such "humanized" antibodies may include antibodies wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some framework residues are substituted by residues from analogous sites in rodent antibodies. See, for example, U.S. Patent Nos. 5,225,539; 5,585,089; 5,693,761;

5,693,762; 5,859,205. See also U.S. Patent No. 6,180,370, and International Publication No. WO 01/27160, where humanized antibodies and techniques for producing humanized antibodies having improved affinity for a predetermined antigen are disclosed.

Also encompassed by the term anti-CD40 antibodies are xenogeneic or modified anti-CD40 antibodies produced in a non-human mammalian host, more particularly a transgenic mouse, characterized by inactivated endogenous immunoglobulin (Ig) loci. In such transgenic animals, competent endogenous genes for the expression of light and heavy subunits of host immunoglobulins are rendered non-functional and substituted with the analogous human immunoglobulin loci. These transgenic animals produce human antibodies in the substantial absence of light or heavy host immunoglobulin subunits. See, for example, U.S. Patent Nos. 5,877,397 and 5,939,598, herein incorporated by reference.

Preferably, fully human antibodies to CD40 are obtained by immunizing transgenic mice. One such mouse is referred to as a Xenomouse, and is disclosed in U.S. Patent Nos. 6,075,181, 6,091,001, and 6,114,598, all of which are incorporated herein by reference. To produce the antibodies disclosed herein, mice transgenic for the human Ig G₁ heavy chain locus and the human K light chain locus were immunized with Sf 9 cells expressing human CD40. Mice can also be transgenic for other isotypes. Fully human antibodies useful in the methods of the present invention are characterized by binding properties similar to those exhibited by the 5.9 and 12.12 monoclonal antibodies disclosed herein.

Fragments of the anti-CD40 antibodies are suitable for use in the methods of the invention so long as they retain the desired affinity of the full-length antibody. Thus, a fragment of an anti-CD40 antibody will retain the ability to bind to the CD40 B cell surface antigen. Such fragments are characterized by properties similar to the corresponding full-length antagonist anti-CD40 antibody, that is, the fragments will specifically bind a human CD40 antigen expressed on the surface of a human cell, and are free of significant agonist activity but exhibit antagonist activity when bound to a CD40 antigen on a human CD40-expressing cell. Such fragments are referred to herein as "antigen-binding" fragments.

Suitable antigen-binding fragments of an antibody comprise a portion of a full-length antibody, generally the antigen-binding or variable region thereof. Examples of antibody fragments include, but are not limited to, Fab, F(ab')₂, and Fv fragments and single-chain antibody molecules. By "Fab" is intended a monovalent antigen-binding fragment of an immunoglobulin that is composed of the light chain and part of the heavy chain. By F(ab')₂ is intended a bivalent antigen-binding fragment of an immunoglobulin that contains both light chains and part of both heavy chains. By "single-chain Fv" or "sFv" antibody fragments is intended fragments comprising the V_H and V_L domains of an antibody, wherein these domains are present in a single polypeptide chain. See, for example, U.S. Patent Nos. 4,946,778, 5,260,203, 5,455,030, and 5,856,456, herein incorporated by reference. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains that enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun (1994) in *The Pharmacology of Monoclonal Antibodies*, Vol. 113, ed. Rosenberg and Moore (Springer-Verlag, New York), pp. 269-315. Antigen-binding fragments of the antagonist anti-CD40 antibodies disclosed herein can also be conjugated to a cytotoxic toxin to effect killing of the target cancer cells, as described herein below.

Antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in, for example, McCafferty *et al.* (1990) *Nature* 348:552-554 (1990) and U.S. Patent No. 5,514,548. Clackson *et al.* (1991) *Nature* 352:624-628 and Marks *et al.* (1991) *J. Mol. Biol.* 222:581-597 describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks *et al.* (1992) *Bio/Technology* 10:779-783), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse *et al.* (1993) *Nucleic. Acids Res.* 21:2265-2266). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived *via* proteolytic digestion of intact antibodies (see, e.g., Morimoto *et al.* (1992) *Journal of Biochemical and Biophysical*

Methods 24:107-117 (1992) and Brennan *et al.* (1985) *Science* 229:81). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form F(ab')₂ fragments (Carter *et al.* (1992) *Bio/Technology* 10:163-167). According to another approach, F(ab')₂ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner.

Antagonist anti-CD40 antibodies useful in the methods of the present invention include the 5.9 and 12.12 monoclonal antibodies disclosed herein as well as antibodies differing from this antibody but retaining the CDRs; and antibodies with one or more amino acid addition(s), deletion(s), or substitution(s), wherein the antagonist activity is measured by inhibition of B-cell proliferation and/or differentiation. The invention also encompasses de-immunized antagonist anti-CD40 antibodies, which can be produced as described in, for example, International Publication Nos. WO 98/52976 and WO 0034317; herein incorporated by reference. In this manner, residues within the antagonist anti-CD40 antibodies of the invention are modified so as to render the antibodies non- or less immunogenic to humans while retaining their antagonist activity toward human CD40-expressing cells, wherein such activity is measured by assays noted elsewhere herein. Also included within the scope of the claims are fusion proteins comprising an antagonist anti-CD40 antibody of the invention, or a fragment thereof, which fusion proteins can be synthesized or expressed from corresponding polynucleotide vectors, as is known in the art. Such fusion proteins are described with reference to conjugation of antibodies as noted below.

The antibodies of the present invention can have sequence variations produced using methods described in, for example, Patent Publication Nos. EP 0 983 303 A1, WO 00/34317, and WO 98/52976, incorporated herein by reference. For example, it has been shown that sequences within the CDR can cause an antibody to bind to MHC Class II and trigger an unwanted helper T-cell response. A conservative substitution can allow the antibody to retain binding activity yet lose its ability to trigger an unwanted T-cell response. Any such conservative or non-conservative substitutions can be made using

art-recognized methods, such as those noted elsewhere herein, and the resulting antibodies will fall within the scope of the invention. The variant antibodies can be routinely tested for antagonist activity, affinity, and specificity using methods described herein.

5 An antibody produced by any of the methods described above, or any other method not disclosed herein, will fall within the scope of the invention if it possesses at least one of the following biological activities: inhibition of immunoglobulin secretion by normal human peripheral B cells stimulated by T cells; inhibition of proliferation of normal human peripheral B cells stimulated by Jurkat T cells; inhibition of proliferation
10 of normal human peripheral B cells stimulated by CD40L-expressing cells or soluble CD40; and inhibition of proliferation of human malignant B cells as noted below. These assays can be performed as described in the Examples herein. See also the assays described in Schultze *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 92:8200-8204; Denton *et al.* (1998) *Pediatr. Transplant.* 2:6-15; Evans *et al.* (2000) *J. Immunol.* 164:688-697;
15 Noelle (1998) *Agents Actions Suppl.* 49:17-22; Lederman *et al.* (1996) *Curr. Opin. Hematol.* 3:77-86; Coligan *et al.* (1991) *Current Protocols in Immunology* 13:12; Kwekkeboom *et al.* (1993) *Immunology* 79:439-444; and U.S. Patent Nos. 5,674,492 and 5,847,082; herein incorporated by reference.

A representative assay to detect antagonistic anti-CD40 antibodies specific to the
20 CD40-antigen epitopes identified herein is a "competitive binding assay." Competitive binding assays are serological assays in which unknowns are detected and quantitated by their ability to inhibit the binding of a labeled known ligand to its specific antibody. This is also referred to as a competitive inhibition assay. In a representative competitive binding assay, labeled CD40 polypeptide is precipitated by candidate antibodies in a
25 sample, for example, in combination with monoclonal antibodies raised against one or more epitopes of the monoclonal antibodies of the invention. Anti-CD40 antibodies that specifically react with an epitope of interest can be identified by screening a series of antibodies prepared against a CD40 protein or fragment of the protein comprising the particular epitope of the CD40 protein of interest. For example, for human CD40,
30 epitopes of interest include epitopes comprising linear and/or nonlinear amino acid residues of the short isoform of human CD40 (see GenBank Accession No. NP_690593)

set forth in Figure 12B (SEQ ID NO:10), encoded by the sequence set forth in Figure 12A (SEQ ID NO:9; see also GenBank Accession No. NM_152854), or of the long isoform of human CD40 (see GenBank Accession Nos. CAA43045 and NP_001241) set forth in Figure 12D (SEQ ID NO:12), encoded by the sequence set forth in Figure 12C (SEQ ID NO:11; see GenBank Accession Nos. X60592 and NM_001250). Alternatively, competitive binding assays with previously identified suitable antagonist anti-CD40 antibodies could be used to select monoclonal antibodies comparable to the previously identified antibodies.

Antibodies employed in such immunoassays may be labeled or unlabeled.

10 Unlabeled antibodies may be employed in agglutination; labeled antibodies may be employed in a wide variety of assays, employing a wide variety of labels. Detection of the formation of an antibody-antigen complex between an anti-CD40 antibody and an epitope of interest can be facilitated by attaching a detectable substance to the antibody. Suitable detection means include the use of labels such as radionuclides, enzymes, 15 coenzymes, fluorescers, chemiluminescers, chromogens, enzyme substrates or co-factors, enzyme inhibitors, prosthetic group complexes, free radicals, particles, dyes, and the like. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials 20 include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material is luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin; and examples of suitable radioactive material include ^{125}I , ^{131}I , ^{35}S , or ^3H . Such labeled reagents may be used in a variety of well-known assays, such as 25 radioimmunoassays, enzyme immunoassays, e.g., ELISA, fluorescent immunoassays, and the like. See for example, U.S. Patent Nos. 3,766,162; 3,791,932; 3,817,837; and 4,233,402.

Any of the previously described antagonist anti-CD40 antibodies or antibody fragments thereof may be conjugated prior to use in the methods of the present invention. 30 Methods for producing conjugated antibodies are known in the art. Thus, the anti-CD40 antibody may be labeled using an indirect labeling or indirect labeling approach. By

"indirect labeling" or "indirect labeling approach" is intended that a chelating agent is covalently attached to an antibody and at least one radionuclide is inserted into the chelating agent. See, for example, the chelating agents and radionuclides described in Srivagtava and Mease (1991) *Nucl. Med. Bio.* 18:589-603, herein incorporated by reference. Suitable labels include fluorophores, chromophores, radioactive atoms (particularly ^{32}P and ^{125}I), electron-dense reagents, enzymes, and ligands having specific binding partners. Enzymes are typically detected by their activity. For example, horseradish peroxidase is usually detected by its ability to convert 3,3',5,5'-tetramethylbenzidine (TMB) to a blue pigment, quantifiable with a spectrophotometer.

"Specific binding partner" refers to a protein capable of binding a ligand molecule with high specificity, as for example in the case of an antigen and a monoclonal antibody specific therefore. Other specific binding partners include biotin and avidin or streptavidin, Ig G and protein A, and the numerous receptor-ligand couples known in the art. It should be understood that the above description is not meant to categorize the various labels into distinct classes, as the same label may serve in several different modes. For example, ^{125}I may serve as a radioactive label or as an electron-dense reagent. HRP may serve as enzyme or as antigen for a mAb. Further, one may combine various labels for desired effect. For example, mAbs and avidin also require labels in the practice of this invention: thus, one might label a mAb with biotin, and detect its presence with avidin labeled with ^{125}I , or with an anti-biotin mAb labeled with HRP. Other permutations and possibilities will be readily apparent to those of ordinary skill in the art, and are considered as equivalents within the scope of the instant invention.

Alternatively, the anti-CD40 antibody may be labeled using "direct labeling" or a "direct labeling approach," where a radionuclide is covalently attached directly to an antibody (typically via an amino acid residue). Preferred radionuclides are provided in Srivagtava and Mease (1991) *supra*. The indirect labeling approach is particularly preferred. See also, for example, International Publication Nos. WO 00/52031 and WO 00/52473, where a linker is used to attach a radioactive label to antibodies; and the labeled forms of anti-CD40 antibodies described in U.S. Patent No. 6,015,542; herein incorporated by reference.

Further, an antibody (or fragment thereof) may be conjugated to a therapeutic moiety such as a cytotoxin, a therapeutic agent, or a radioactive metal ion. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine and vinblastine). The conjugates of the invention can be used for modifying a given biological response; the drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor, interferon-alpha, interferon-beta, nerve growth factor, platelet derived growth factor, tissue plasminogen activator; or, biological response modifiers such as, for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-CSF"), granulocyte colony stimulating factor ("G-CSF"), or other growth factors.

Techniques for conjugating such therapeutic moiety to antibodies are well known. See, for example, Arnon *et al.* (1985) "Monoclonal Antibodies for Immunotargeting of Drugs in Cancer Therapy," in *Monoclonal Antibodies and Cancer Therapy*, ed. Reisfeld *et al.* (Alan R. Liss, Inc.), pp. 243-256; ed. Hellstrom *et al.* (1987) "Antibodies for Drug Delivery," in *Controlled Drug Delivery*, ed. Robinson *et al.* (2d ed; Marcel Dekker, Inc.),

pp. 623-653; Thorpe (1985) "Antibody Carriers of Cytotoxic Agents in Cancer Therapy: A Review," in *Monoclonal Antibodies '84: Biological and Clinical Applications*, ed. Pinchera *et al.* pp. 475-506 (Editrice Kurtis, Milano, Italy, 1985); "Analysis, Results, and Future Prospective of the Therapeutic Use of Radiolabeled Antibody in Cancer Therapy,"
5 in *Monoclonal Antibodies for Cancer Detection and Therapy*, ed. Baldwin *et al.* (Academic Press, New York, 1985), pp. 303-316; and Thorpe *et al.* (1982) *Immunol. Rev.* 62:119-158.

Alternatively, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate as described in U.S. Patent No. 4,676,980. In addition, linkers
10 may be used between the labels and the antibodies of the invention (see U.S. Patent No. 4,831,175). Antibodies or, antigen-binding fragments thereof may be directly labeled with radioactive iodine, indium, yttrium, or other radioactive particle known in the art (U.S. Patent No. 5,595,721). Treatment may consist of a combination of treatment with conjugated and nonconjugated antibodies administered simultaneously or subsequently
15 (WO 00/52031 and WO 00/52473).

Variants of Antagonist Anti-CD40 Antibodies

Suitable biologically active variants of the antagonist anti-CD40 antibodies can be used in the methods of the present invention. Such variants will retain the desired
20 binding properties of the parent antagonist anti-CD40 antibody. Methods for making antibody variants are generally available in the art.

For example, amino acid sequence variants of an antagonist anti-CD40 antibody, for example, the 5.9 or 12.12 monoclonal antibody described herein, can be prepared by mutations in the cloned DNA sequence encoding the antibody of interest. Methods for
25 mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York); Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel *et al.* (1987) *Methods Enzymol.* 154:367-382; Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor, New York); U.S. Patent No.
30 4,873,192; and the references cited therein; herein incorporated by reference. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the

polypeptide of interest may be found in the model of Dayhoff *et al.* (1978) in *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be preferred. Examples of conservative substitutions include, but are not limited to, Gly \leftrightarrow Ala, Val \leftrightarrow Ile \leftrightarrow Leu, Asp \leftrightarrow Glu, Lys \leftrightarrow Arg, Asn \leftrightarrow Gln, and Phe \leftrightarrow Trp \leftrightarrow Tyr.

In constructing variants of the antagonist anti-CD40 antibody polypeptide of interest, modifications are made such that variants continue to possess the desired activity, i.e., similar binding affinity and are capable of specifically binding to a human CD40 antigen expressed on the surface of a human cell, and being free of significant agonist activity but exhibiting antagonist activity when bound to a CD40 antigen on a human CD40-expressing cell. Obviously, any mutations made in the DNA encoding the variant polypeptide must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. See EP Patent Application Publication No. 75,444.

Preferably, variants of a reference antagonist anti-CD40 antibody have amino acid sequences that have at least 70% or 75% sequence identity, preferably at least 80% or 85% sequence identity, more preferably at least 90%, 91%, 92%, 93%, 94% or 95% sequence identity to the amino acid sequence for the reference antagonist anti-CD40 antibody molecule, for example, the 5.9 or 12.12 monoclonal antibody described herein, or to a shorter portion of the reference antibody molecule. More preferably, the molecules share at least 96%, 97%, 98% or 99% sequence identity. For purposes of the present invention, percent sequence identity is determined using the Smith-Waterman homology search algorithm using an affine gap search with a gap open penalty of 12 and a gap extension penalty of 2, BLOSUM matrix of 62. The Smith-Waterman homology search algorithm is taught in Smith and Waterman (1981) *Adv. Appl. Math.* 2:482-489. A variant may, for example, differ from the reference antagonist anti-CD40 antibody by as few as 1 to 15 amino acid residues, as few as 1 to 10 amino acid residues, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue.

With respect to optimal alignment of two amino acid sequences, the contiguous segment of the variant amino acid sequence may have additional amino acid residues or

deleted amino acid residues with respect to the reference amino acid sequence. The contiguous segment used for comparison to the reference amino acid sequence will include at least 20 contiguous amino acid residues, and may be 30, 40, 50, or more amino acid residues. Corrections for sequence identity associated with conservative residue substitutions or gaps can be made (see Smith-Waterman homology search algorithm).

The precise chemical structure of a polypeptide capable of specifically binding CD40 and retaining antagonist activity, particularly when bound to CD40 antigen on malignant B cells, depends on a number of factors. As ionizable amino and carboxyl groups are present in the molecule, a particular polypeptide may be obtained as an acidic or basic salt, or in neutral form. All such preparations that retain their biological activity when placed in suitable environmental conditions are included in the definition of antagonist anti-CD40 antibodies as used herein. Further, the primary amino acid sequence of the polypeptide may be augmented by derivatization using sugar moieties (glycosylation) or by other supplementary molecules such as lipids, phosphate, acetyl groups and the like. It may also be augmented by conjugation with saccharides. Certain aspects of such augmentation are accomplished through post-translational processing systems of the producing host; other such modifications may be introduced *in vitro*. In any event, such modifications are included in the definition of an anti-CD40 antibody used herein so long as the antagonist properties of the anti-CD40 antibody are not destroyed. It is expected that such modifications may quantitatively or qualitatively affect the activity, either by enhancing or diminishing the activity of the polypeptide, in the various assays. Further, individual amino acid residues in the chain may be modified by oxidation, reduction, or other derivatization, and the polypeptide may be cleaved to obtain fragments that retain activity. Such alterations that do not destroy antagonist activity do not remove the polypeptide sequence from the definition of anti-CD40 antibodies of interest as used herein.

The art provides substantial guidance regarding the preparation and use of polypeptide variants. In preparing the anti-CD40 antibody variants, one of skill in the art can readily determine which modifications to the native protein nucleotide or amino acid sequence will result in a variant that is suitable for use as a therapeutically active component of a pharmaceutical composition used in the methods of the present invention.

Methods of Therapy Using the Antagonist Anti-CD40 Antibodies of the Invention

Methods of the invention are directed to the use of antagonist anti-CD40 antibodies to treat patients having a disease mediated by stimulation of CD40 signaling on CD40-expressing cells. By "CD40-expressing cell" is intended normal and malignant B cells expressing CD40 antigen. By "malignant" B cell is intended any neoplastic B cell, including but not limited to B cells derived from lymphomas including low-, intermediate-, and high-grade B cell lymphomas, immunoblastic lymphomas, non-Hodgkin's lymphomas, Hodgkin's disease, Epstein-Barr Virus (EBV) induced lymphomas, and AIDS-related lymphomas, as well as B cell acute lymphoblastic leukemias, myelomas, chronic lymphocytic leukemias, acute myeloblastic leukemias, and the like.

"Treatment" is herein defined as the application or administration of an antagonist anti-CD40 antibody or antigen-binding fragment thereof to a patient, or application or administration of an antagonist anti-CD40 antibody or fragment thereof to an isolated tissue or cell line from a patient, where the patient has a disease, a symptom of a disease, or a predisposition toward a disease, where the purpose is to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect the disease, the symptoms of the disease, or the predisposition toward the disease. By "treatment" is also intended the application or administration of a pharmaceutical composition comprising the antagonist anti-CD40 antibodies or fragments thereof to a patient, or application or administration of a pharmaceutical composition comprising the anti-CD40 antibodies or fragments thereof to an isolated tissue or cell line from a patient, who has a disease, a symptom of a disease, or a predisposition toward a disease, where the purpose is to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect the disease, the symptoms of the disease, or the predisposition toward the disease.

By "anti-tumor activity" is intended a reduction in the rate of malignant CD40-expressing cell proliferation or accumulation, and hence a decline in growth rate of an existing tumor or in a tumor that arises during therapy, and/or destruction of existing neoplastic (tumor) cells or newly formed neoplastic cells, and hence a decrease in the overall size of a tumor during therapy. Therapy with at least one anti-CD40 antibody (or antigen-binding fragment thereof) causes a physiological response that is beneficial with

respect to treatment of disease states associated with stimulation of CD40 signaling on CD40-expressing cells in a human.

The methods of the invention find use in the treatment of non-Hodgkin's lymphomas related to abnormal, uncontrollable B cell proliferation or accumulation. For purposes of the present invention, such lymphomas will be referred to according to the *Working Formulation* classification scheme, that is those B cell lymphomas categorized as low grade, intermediate grade, and high grade (see "The Non-Hodgkin's Lymphoma Pathologic Classification Project," *Cancer* 49(1982):2112-2135). Thus, low-grade B cell lymphomas include small lymphocytic, follicular small-cleaved cell, and follicular mixed small-cleaved and large cell lymphomas; intermediate-grade lymphomas include follicular large cell, diffuse small cleaved cell, diffuse mixed small and large cell, and diffuse large cell lymphomas; and high-grade lymphomas include large cell immunoblastic, lymphoblastic, and small non-cleaved cell lymphomas of the Burkitt's and non-Burkitt's type.

It is recognized that the methods of the invention are useful in the therapeutic treatment of B cell lymphomas that are classified according to the Revised European and American Lymphoma Classification (REAL) system. Such B cell lymphomas include, but are not limited to, lymphomas classified as precursor B cell neoplasms, such as B lymphoblastic leukemia/lymphoma; peripheral B cell neoplasms, including B cell chronic lymphocytic leukemia/small lymphocytic lymphoma, lymphoplasmacytoid lymphoma/immunocytoma, mantle cell lymphoma (MCL), follicle center lymphoma (follicular) (including diffuse small cell, diffuse mixed small and large cell, and diffuse large cell lymphomas), marginal zone B cell lymphoma (including extranodal, nodal, and splenic types), hairy cell leukemia, plasmacytoma/ myeloma, diffuse large cell B cell lymphoma of the subtype primary mediastinal (thymic), Burkitt's lymphoma, and Burkitt's like high grade B cell lymphoma; acute leukemias; acute lymphocytic leukemias; myeloblastic leukemias; acute myelocytic leukemias; promyelocytic leukemia; myelomonocytic leukemia; monocytic leukemia; erythroleukemia; granulocytic leukemia (chronic myelocytic leukemia); chronic lymphocytic leukemia; polycythemia vera; multiple myeloma; Waldenstrom's macroglobulinemia; heavy chain disease; and unclassifiable low-grade or high-grade B cell lymphomas.

It is recognized that the methods of the invention may be useful in preventing further tumor outgrowths arising during therapy. The methods of the invention are particularly useful in the treatment of subjects having low-grade B cell lymphomas, particularly those subjects having relapses following standard chemotherapy. Low-grade
5 B cell lymphomas are more indolent than the intermediate- and high-grade B cell lymphomas and are characterized by a relapsing/remitting course. Thus, treatment of these lymphomas is improved using the methods of the invention, as relapse episodes are reduced in number and severity.

The antagonist anti-CD40 antibodies described herein may also find use in the
10 treatment of inflammatory diseases and deficiencies or disorders of the immune system including, but not limited to, systemic lupus erythematosus, psoriasis, scleroderma, CREST syndrome, inflammatory myositis, Sjogren's syndrome, mixed connective tissue disease, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, acute respiratory distress syndrome, pulmonary inflammation, idiopathic pulmonary fibrosis,
15 osteoporosis, delayed type hypersensitivity, asthma, primary biliary cirrhosis, and idiopathic thrombocytopenic purpura.

In accordance with the methods of the present invention, at least one antagonist anti-CD40 antibody (or antigen-binding fragment thereof) as defined elsewhere herein is used to promote a positive therapeutic response with respect to a malignant human B cell.
20 By "positive therapeutic response" with respect to cancer treatment is intended an improvement in the disease in association with the anti-tumor activity of these antibodies or fragments thereof, and/or an improvement in the symptoms associated with the disease. That is, an anti-proliferative effect, the prevention of further tumor outgrowths, a reduction in tumor size, a reduction in the number of cancer cells, and/or a decrease in
25 one or more symptoms mediated by stimulation of CD40-expressing cells can be observed. Thus, for example, an improvement in the disease may be characterized as a complete response. By "complete response" is intended an absence of clinically detectable disease with normalization of any previously abnormal radiographic studies, bone marrow, and cerebrospinal fluid (CSF). Such a response must persist for at least
30 one month following treatment according to the methods of the invention. Alternatively, an improvement in the disease may be categorized as being a partial response. By

"partial response" is intended at least about a 50% decrease in all measurable tumor burden (i.e., the number of tumor cells present in the subject) in the absence of new lesions and persisting for at least one month. Such a response is applicable to measurable tumors only.

5 Tumor response can be assessed for changes in tumor morphology (i.e., overall tumor burden, tumor size, and the like) using screening techniques such as magnetic resonance imaging (MRI) scan, x-radiographic imaging, computed tomographic (CT) scan, bioluminescent imaging, for example, luciferase imaging, bone scan imaging, and tumor biopsy sampling including bone marrow aspiration (BMA). In addition to these
10 positive therapeutic responses, the subject undergoing therapy with the antagonist anti-CD40 antibody or antigen-binding fragment thereof may experience the beneficial effect of an improvement in the symptoms associated with the disease. Thus for B cell tumors, the subject may experience a decrease in the so-called B symptoms, i.e., night sweats, fever, weight loss, and/or urticaria.

15 By "therapeutically effective dose or amount" or "effective amount" is intended an amount of antagonist anti-CD40 antibody or antigen-binding fragment thereof that, when administered brings about a positive therapeutic response with respect to treatment of a patient with a disease comprising stimulation of CD40-expressing cells. In some embodiments of the invention, a therapeutically effective dose of the anti-CD40 antibody
20 or fragment thereof is in the range from about 0.01 mg/kg to about 40 mg/kg, from about 0.01 mg/kg to about 30 mg/kg, from about 0.1 mg/kg to about 30 mg/kg, from about 1 mg/kg to about 30 mg/kg, from about 3 mg/kg to about 30 mg/kg, from about 3 mg/kg to about 25 mg/kg, from about 3 mg/kg to about 20 mg/kg, from about 5 mg/kg to about 15 mg/kg, or from about 7 mg/kg to about 12 mg/kg. It is recognized that the method of
25 treatment may comprise a single administration of a therapeutically effective dose or multiple administrations of a therapeutically effective dose of the antagonist anti-CD40 antibody or antigen-binding fragment thereof.

A further embodiment of the invention is the use of antagonist anti-CD40 antibodies for diagnostic monitoring of protein levels in tissue as part of a clinical testing
30 procedure, e.g., to determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling the antibody to a detectable substance. Examples of detectable

substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin; and examples of suitable radioactive material include ^{125}I , ^{131}I , ^{35}S , or ^3H .

The antagonist anti-CD40 antibodies can be used in combination with known chemotherapeutics and cytokines for the treatment of disease states comprising stimulated CD40-expressing cells. For example, the anti-CD40 antibodies of the invention can be used in combination with cytokines such as interleukin-2. In another embodiment, the anti-CD40 antibodies of the invention can be used in combination with rituximab (IDEC-C2B8; Rituxan®; IDEC Pharmaceuticals Corp., San Diego, California).

The anti-CD40 antibodies described herein can further be used to provide reagents, e.g., labeled antibodies that can be used, for example, to identify cells expressing CD40. This can be very useful in determining the cell type of an unknown sample. Panels of monoclonal antibodies can be used to identify tissue by species and/or by organ type. In a similar fashion, these anti-CD40 antibodies can be used to screen tissue culture cells for contamination (i.e., screen for the presence of a mixture of CD40-expressing and non-CD40 expressing cells in a culture).

Pharmaceutical Formulations and Modes of Administration

The antagonist anti-CD40 antibodies of this invention are administered at a concentration that is therapeutically effective to prevent or treat CD40-expressing cell-mediated diseases such as SLE, PBC, ITP, multiple sclerosis, psoriasis, Crohn's disease, graft rejection, and B-cell lymphoma. To accomplish this goal, the antibodies may be formulated using a variety of acceptable excipients known in the art. Typically, the antibodies are administered by injection, either intravenously or intraperitoneally. Methods to accomplish this administration are known to those of ordinary skill in the art.

It may also be possible to obtain compositions which may be topically or orally administered, or which may be capable of transmission across mucous membranes.

Intravenous administration occurs preferably by infusion over a period of about 1 to about 10 hours, more preferably over about 1 to about 8 hours, even more preferably over about 2 to about 7 hours, still more preferably over about 4 to about 6 hours, depending upon the anti-CD40 antibody being administered. The initial infusion with the pharmaceutical composition may be given over a period of about 4 to about 6 hours with subsequent infusions delivered more quickly. Subsequent infusions may be administered over a period of about 1 to about 6 hours, including, for example, about 1 to about 4 hours, about 1 to about 3 hours, or about 1 to about 2 hours.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of possible routes of administration include parenteral, (e.g., intravenous (IV), intramuscular (IM), intradermal, subcutaneous (SC), or infusion), oral and pulmonary (e.g., inhalation), nasal, transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerin, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes, or multiple dose vials made of glass or plastic.

The anti-CD40 antibodies are typically provided by standard technique within a pharmaceutically acceptable buffer, for example, sterile saline, sterile buffered water, propylene glycol, combinations of the foregoing, etc. Methods for preparing parenterally administrable agents are described in *Remington's Pharmaceutical Sciences* (18th ed.; Mack Publishing Company, Eaton, Pennsylvania, 1990), herein incorporated by reference. See also, for example, WO 98/56418, which describes stabilized antibody pharmaceutical formulations suitable for use in the methods of the present invention.

The amount of at least one anti-CD40 antibody or fragment thereof to be administered is readily determined by one of ordinary skill in the art without undue experimentation. Factors influencing the mode of administration and the respective amount of at least one antagonist anti-CD40 antibody (or fragment thereof) include, but are not limited to, the the severity of the disease, the history of the disease, and the age, height, weight, health, and physical condition of the individual undergoing therapy. Similarly, the amount of antagonist anti-CD40 antibody or fragment thereof to be administered will be dependent upon the mode of administration and whether the subject will undergo a single dose or multiple doses of this anti-tumor agent. Generally, a higher dosage of anti-CD40 antibody or fragment thereof is preferred with increasing weight of the subject undergoing therapy. The dose of anti-CD40 antibody or fragment thereof to be administered is in the range from about 0.003 mg/kg to about 50 mg/kg, preferably in the range of 0.01 mg/kg to about 40 mg/kg. Thus, for example, the dose can be 0.01 mg/kg, 0.03 mg/kg, 0.1 mg/kg, 0.3 mg/kg, 0.5 mg/kg, 1 mg/kg, 1.5 mg/kg, 2 mg/kg, 2.5 mg/kg, 3 mg/kg, 5 mg/kg, 7 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg, 25 mg/kg, 30 mg/kg, 35 mg/kg, 40 mg/kg, 45 mg/kg, or 50 mg/kg.

In another embodiment of the invention, the method comprises administration of multiple doses of antagonist anti-CD40 antibody or fragment thereof. The method may comprise administration of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, or more therapeutically effective doses of a pharmaceutical composition comprising an antagonist anti-CD40 antibody or fragment thereof. The frequency and duration of administration of multiple doses of the pharmaceutical compositions comprising anti-CD40 antibody or fragment thereof can be readily determined by one of skill in the art without undue experimentation. Moreover, treatment of a subject with a therapeutically effective amount of an antibody can include a single treatment or, preferably, can include a series of treatments. In a preferred example, a subject is treated with antagonist anti-CD40 antibody or antigen-binding fragment thereof in the range of between about 0.1 to 20 mg/kg body weight, once per week for between about 1 to 10 weeks, preferably between about 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. Treatment may occur annually to prevent relapse or upon indication of relapse. It will also be appreciated that the effective dosage of

antibody or antigen-binding fragment thereof used for treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result and become apparent from the results of diagnostic assays as described herein. Thus, in one embodiment, the dosing regimen includes a first administration of a therapeutically effective dose of at least one anti-CD40 antibody or fragment thereof on days 1, 7, 14, and 21 of a treatment period. In another embodiment, the dosing regimen includes a first administration of a therapeutically effective dose of at least one anti-CD40 antibody or fragment thereof on days 1, 2, 3, 4, 5, 6, and 7 of a week in a treatment period. Further embodiments include a dosing regimen having a first administration of a therapeutically effective dose of at least one anti-CD40 antibody or fragment thereof on days 1, 3, 5, and 7 of a week in a treatment period; a dosing regimen including a first administration of a therapeutically effective dose of at least one anti-CD40 antibody or fragment thereof on days 1 and 3 of a week in a treatment period; and a preferred dosing regimen including a first administration of a therapeutically effective dose of at least one anti-CD40 antibody or fragment thereof on day 1 of a week in a treatment period. The treatment period may comprise 1 week, 2 weeks, 3 weeks, a month, 3 months, 6 months, or a year. Treatment periods may be subsequent or separated from each other by a day, a week, 2 weeks, a month, 3 months, 6 months, or a year.

The antagonist anti-CD40 antibodies present in the pharmaceutical compositions described herein for use in the methods of the invention may be native or obtained by recombinant techniques, and may be from any source, including mammalian sources such as, e.g., mouse, rat, rabbit, primate, pig, and human. Preferably such polypeptides are derived from a human source, and more preferably are recombinant, human proteins from hybridoma cell lines.

The pharmaceutical compositions useful in the methods of the invention may comprise biologically active variants of the antagonist anti-CD40 antibodies of the invention. Such variants should retain the desired biological activity of the native polypeptide such that the pharmaceutical composition comprising the variant polypeptide has the same therapeutic effect as the pharmaceutical composition comprising the native polypeptide when administered to a subject. That is, the variant anti-CD40 antibody will serve as a therapeutically active component in the pharmaceutical composition in a

manner similar to that observed for the native antagonist antibody, for example 5.9 or 12.12 as expressed by the hybridoma cell line 5.9 or 12.12, respectively. Methods are available in the art for determining whether a variant anti-CD40 antibody retains the desired biological activity, and hence serves as a therapeutically active component in the pharmaceutical composition. Biological activity of antibody variants can be measured using assays specifically designed for measuring activity of the native antagonist antibody, including assays described in the present invention.

Any pharmaceutical composition comprising an antagonist anti-CD40 antibody having the binding properties described herein as the therapeutically active component can be used in the methods of the invention. Thus liquid, lyophilized, or spray-dried compositions comprising one or more of the antagonist anti-CD40 antibodies of the invention may be prepared as an aqueous or nonaqueous solution or suspension for subsequent administration to a subject in accordance with the methods of the invention. Each of these compositions will comprise at least one of the antagonist anti-CD40 antibodies of the present invention as a therapeutically or prophylactically active component. By "therapeutically or prophylactically active component" is intended the anti-CD40 antibody is specifically incorporated into the composition to bring about a desired therapeutic or prophylactic response with regard to treatment, prevention, or diagnosis of a disease or condition within a subject when the pharmaceutical composition is administered to that subject. Preferably the pharmaceutical compositions comprise appropriate stabilizing agents, bulking agents, or both to minimize problems associated with loss of protein stability and biological activity during preparation and storage.

Formulants may be added to pharmaceutical compositions comprising an antagonist anti-CD40 antibody of the invention. These formulants may include, but are not limited to, oils, polymers, vitamins, carbohydrates, amine acids, salts, buffers, albumin, surfactants, or bulking agents. Preferably carbohydrates include sugar or sugar alcohols such as mono-, di-, or polysaccharides, or water soluble glucans. The saccharides or glucans can include fructose, glucose, mannose, sorbose, xylose, maltose, sucrose, dextran, pullulan, dextrin, α and β cyclodextrin, soluble starch, hydroxyethyl starch, and carboxymethylcellulose, or mixtures thereof. "Sugar alcohol" is defined as a C₄ to C₈ hydrocarbon having a hydroxyl group and includes galactitol, inositol, mannitol,

xylitol, sorbitol, glycerol, and arabitol. These sugars or sugar alcohols may be used individually or in combination. The sugar or sugar alcohol concentration is between 1.0% and 7% w/v., more preferably between 2.0% and 6.0% w/v. Preferably amino acids include levorotary (L) forms of carnitine, arginine, and betaine; however, other amino acids may be added. Preferred polymers include polyvinylpyrrolidone (PVP) with an average molecular weight between 2,000 and 3,000, or polyethylene glycol (PEG) with an average molecular weight between 3,000 and 5,000. Surfactants that can be added to the formulation are shown in EP Nos. 270,799 and 268,110.

Additionally, antibodies can be chemically modified by covalent conjugation to a polymer to increase their circulating half-life, for example. Preferred polymers, and methods to attach them to peptides, are shown in U.S. Patent Nos. 4,766,106; 4,179,337; 4,495,285; and 4,609,546; which are all hereby incorporated by reference in their entirety. Preferred polymers are polyoxyethylated polyols and polyethylene glycol (PEG). PEG is soluble in water at room temperature and has the general formula: $R(O-CH_2-CH_2)_nO-R$ where R can be hydrogen, or a protective group such as an alkyl or alkanol group. Preferably, the protective group has between 1 and 8 carbons, more preferably it is methyl. The symbol n is a positive integer, preferably between 1 and 1,000, more preferably between 2 and 500. The PEG has a preferred average molecular weight between 1,000 and 40,000, more preferably between 2,000 and 20,000, most preferably between 3,000 and 12,000. Preferably, PEG has at least one hydroxy group, more preferably it is a terminal hydroxy group. It is this hydroxy group which is preferably activated to react with a free amino group on the inhibitor. However, it will be understood that the type and amount of the reactive groups may be varied to achieve a covalently conjugated PEG/antibody of the present invention.

Water-soluble polyoxyethylated polyols are also useful in the present invention. They include polyoxyethylated sorbitol, polyoxyethylated glucose, polyoxyethylated glycerol (POG), and the like. POG is preferred. One reason is because the glycerol backbone of polyoxyethylated glycerol is the same backbone occurring naturally in, for example, animals and humans in mono-, di-, triglycerides. Therefore, this branching would not necessarily be seen as a foreign agent in the body. The POG has a preferred molecular weight in the same range as PEG. The structure for POG is shown in Knauf *et*

al. (1988) *J. Bio. Chem.* 263:15064-15070, and a discussion of POG/IL-2 conjugates is found in U.S. Patent No. 4,766,106, both of which are hereby incorporated by reference in their entireties.

Another drug delivery system for increasing circulatory half-life is the liposome.

5 Methods of preparing liposome delivery systems are discussed in Gabizon *et al.* (1982) *Cancer Research* 42:4734; Cafiso (1981) *Biochem Biophys Acta* 649:129; and Szoka (1980) *Ann. Rev. Biophys. Eng.* 9:467. Other drug delivery systems are known in the art and are described in, *e.g.*, Poznansky *et al.* (1980) *Drug Delivery Systems* (R.L. Juliano, ed., Oxford, N.Y.) pp. 253-315; Poznansky (1984) *Pharm Revs* 36:277.

10 After the liquid pharmaceutical composition is prepared, it is preferably lyophilized to prevent degradation and to preserve sterility. Methods for lyophilizing liquid compositions are known to those of ordinary skill in the art. Just prior to use, the composition may be reconstituted with a sterile diluent (Ringer's solution, distilled water, or sterile saline, for example) that may include additional ingredients. Upon
15 reconstitution, the composition is preferably administered to subjects using those methods that are known to those skilled in the art.

The following examples are offered by way of illustration and not by way of limitation.

20 EXPERIMENTAL

The antagonist anti-CD40 antibodies used in the examples below are 5.9 and 12.12. The 5.9 and 12.12 anti-CD40 antibodies are human IgG₁ subtype anti-human CD40 monoclonal antibodies (mAbs) generated by immunization of transgenic mice bearing the human IgG₁ heavy chain locus and the human K light chain locus
25 (Xenomouse, Abgenix). As shown by FACS analysis, 5.9 and 12.12 bind specifically to human CD40 and can prevent CD40 ligand binding. Both mAbs can compete off CD40-ligand pre-bound to cell surface CD40. Both antibodies are strong antagonists and inhibit *in vitro* CD40 ligand-mediated proliferation of normal B cells, as well as cancer cells from NHL and CLL patients. *In vitro*, both antibodies kill cancer cell lines as well as
30 primary cancer cells from NHL patients by ADCC. Dose-dependent anti-tumor activity

was seen in a xenograft human lymphoma model. The binding affinity of 5.9 to human CD40 is 1.2×10^{-8} M and the binding affinity of 12.12 to human CD40 is 5×10^{-10} M.

Mouse hybridoma line 131.2F8.5.9 (CMCC#12047) and hybridoma line 153.8E2.D10.D6.12.12 (CMCC#12056) have been deposited with the American Type Culture Collection [ATCC; 10801 University Blvd., Manassas, Virginia 20110-2209 (USA)] under Patent Deposit Number PTA-S542 and PTA-5543, respectively.

The following protocols have been used in the examples described below.

ELISA Assay for Immunoglobulin Quantification

10 The concentrations of human IgM and IgG were estimated by ELISA. 96-well ELISA plates were coated with 2 µg/ml goat anti-human IgG MAb (The Jackson Laboratory, Bar Harbor, Maine) or with 2 µg/ml goat anti-human IgM MAb 4102 (Bio Source International, California) in 0.05 M carbonate buffer (pH 9.6), by incubation for 16 hours at 4°C. Plates were washed 3 times with PBS-0.05 % Tween-20 (PBS-Tween) and saturated with BSA for 1 hour. After 2 washes the plates were incubated for 2 hour at 37°C with different dilutions of the test samples. After 3 washes, bound Ig was detected by incubation for 2 hour at 37°C with 1 µg/ml peroxidase-labeled goat anti-human IgG MAb or goat anti-human IgM Mab. Plates were washed 4 times, and bound peroxidase activity was revealed by the addition of O-phenylenediamine as a substrate. Human IgG or IgM standards (Caltaq, Burlingame, California) was used to establish a standard curve for each assay.

Isolation of the Peripheral Blood Mononuclear Cells (PBMC) from Human Peripheral Blood

25 20 ml of Ficoll-Paque solution (low endotoxin; Pharmacia) was added per 50 ml polystyrene tube, in 3 tubes, 30 minutes before adding the blood. The Ficoll-Paque solution was warmed up to room temperature. 3 L of bleach in 1:10 dilution was prepared, and used to wash all the tubes and pipettes contacting the blood. The blood was layered on the top of the Ficoll-Paque solution without disturbing the Ficoll layer, at 1.5 ml blood/1 ml of Ficoll-Paque. The tubes were centrifuged at 1700 rpm for 30 minutes at room temperature with the brake on the centrifuge turned off. As much of the

top layer (plasma) as possible was removed, minimizing the vacuum in order to avoid removing the second layer of solution. The second layer, which contains the B and T lymphocytes, was collected using a sterile Pasteur pipette, and placed in two 50-ml polystyrene tubes. The collection was diluted with 3 x the volume of cold RPMI with no additives, and the tubes were centrifuged at 1000 RPM for 10 minutes. The media was removed by aspiration, and the cells from both 50-ml tubes were resuspended in a total of 10 ml cold RPMI (with additives) and transferred to a 15-ml tube. The cells were counted using the hemacytometer, then centrifuged at 1000 RPM for 10 minutes. The media was removed and the cells resuspended in 4 ml RPMI. This fraction contained the PBMC.

Isolation of the B cells from PBMC

100 µl of Dynabeads (anti-h CD19) were placed in a 5-ml plastic tube. 3 ml of sterile PBS were added to the beads and mixed, and placed in the magnetic holder, then allowed to sit for 2 minutes. The solution was removed using a Pasteur pipette. 3 ml of sterile PBS were added, mixed, and placed in the magnetic holder, then allowed to sit for 2 minutes. This procedure with sterile PBS was repeated one more time for a total of 3 washes. The PBMC was added into the beads and incubated, while mixing, for 30 minutes at 40°C. The tube containing the PBMC and beads was placed into the magnetic holder for 2 minutes, then the solution was transferred to a new 5-ml tube in the magnetic holder. After 2 minutes, the solution was transferred to a new 15-ml tube. This step was repeated four more times, and the solutions of the first four times were collected in the 15-ml tube, then centrifuged at 1000 RPM for 5 minutes. This step produced the pellet for T-cell separation.

100 µl RPMI (with additives) was added to collect the beads, and the solution was transferred into a 0.7-ml tube. 10 µl of Dynal Detacha Beads were added into the suspension at room temperature, and it was allowed to rotate for 45 minutes. The suspension was transferred into a new 5-ml tube and 3-ml of RPMI (with additives) were added. The tube was placed in the magnetic holder for 2 minutes. The solution was transferred into a new 5-ml tube in the holder for 2 minutes, then to a 15-ml tube. The previous step was repeated three more times, collecting the solution in the 15-ml tube.

The 15-ml tube was centrifuged at 1000 RPM for 10 minutes, and the cells resuspended in 10 ml RPMI. The washing step was repeated 2 more times for a total of 3 washes. The cells were counted before the last centrifugation. This step completed the B-cell purification. Cells were stored in 90% FCS and 10% DMSO and frozen at -80°C.

5

Isolation of the T Cells

The human T cell Enrichment Column (R&D systems, anti-h CD 3 column kit) was prepared using 20 ml of 1 X column wash buffer by mixing 2 ml of 10 X column wash buffer and 18 ml of sterile distilled water. The column was cleaned with 70% ethanol and placed on top of a 15-ml tube. The top cap of the column was removed first to avoid drawing air into the bottom of the column. Next, the bottom cap was removed, and the tip was cleaned with 70% ethanol. The fluid within the column was allowed to drain into the 15-ml tube. A new sterile 15-ml tube was placed under the column after the column buffer had drained to the level of the white filter. The B-cell depleted PBMC fraction was suspended in 1 ml of buffer and added to the top of the column. The cells were allowed to incubate with the column at room temperature for 10 minutes. The T-cells were eluted from the column with 4 aliquots of 2 ml each of 1 X column wash buffer. The collected T-cells were centrifuged at 1000 RPM for 5 minutes. The supernatant was removed and the cells resuspended in 10 ml RPMI. Cells were counted and centrifuged one more time. The supernatant was removed, completing the T-cell purification. Cells were stored in 90% FCS and 10% DMSO and frozen at -80°C.

For the above procedures, the RPMI composition contained 10% FCS (inactivated at 56°C for 45 min), 1 % Pen/Strep (100 u/ml Penicillin, 0.1 µg/ml Streptomycin), 1% Glutamate, 1% sodium puravate, 50 µM 2-ME.

25

Flow Cytofluorometric Assay

Ramos cells (106 cells/sample) were incubated in 100 µl primary antibody (10 µg/ml in PBS-BSA) for 20 min at 4°C. After 3 washes with PBS-BSA or HBSS- BSA, the cells were incubated in 100 µl FITC-labeled F(ab')₂ fragments of goat anti-(human IgG) antibodies (Caltag) for 20 min at 4°C. After 3 washes with PBS-BSA and 1 wash

30

with PBS, the cells were resuspended in 0.5-ml PBS. Analyses were performed with a FACSCAN V (Becton Dickinson, San Jose, California).

Generation of Hybridoma Clones

5 Splenocytes from immunized mice were fused with SP 2/0 or P 3 x 63Ag8.653 murine myeloma cells at a ratio of 10:1 using 50% polyethylene glycol as previously described by de Boer *et al.* (1988) *J. Immunol. Meth.* 113:143. The fused cells were resuspended in complete IMDM medium supplemented with hypoxanthine (0.1 mM), aminopterin (0.01 mM), thymidine (0.016 mM), and 0.5 ng/ml hIL-6 (Genzyme,
10 Cambridge, Massachusetts). The fused cells were then distributed between the wells of 96-well tissue culture plates, so that each well contained 1 growing hybridoma on average.

 After 10-14 days the supernatants of the hybridoma populations were screened for specific antibody production. For the screening of specific antibody production by the
15 hybridoma clones, the supernatants from each well were pooled and tested for anti-CD 40 activity specificity by ELISA first. The positives were then used for fluorescent cell staining of EBV-transformed B cells as described for the FACS assay above. Positive hybridoma cells were cloned twice by limiting dilution in IMDM/FBS containing 0.5
20 ng/ml hIL-6.

Example 1: Production of Anti-CD40 Antibodies

 Several fully human, antagonistic anti-CD40 monoclonal antibodies of IgG1 isotype were generated. Transgenic mice bearing the human IgG1 heavy chain locus and the human κ chain locus (Abgenix γ -1 xenomouse) were used to generate these
25 antibodies. SF9 insect cells expressing CD40 extracellular domain were used as immunogen. A total of 31 mice spleens were fused with the mouse myeloma SP2/0 cells to generate 895 antibodies that recognize recombinant CD40 in ELISA (Tables 1A and 1B). On average approximately 10% of hybridomas produced in Abgenix xenomice may contain mouse lambda light chain instead of human kappa chain. The antibodies
30 containing mouse light lambda chain were selected out. A subset of 260 antibodies that also showed binding to cell-surface CD40 were selected for further analysis. Stable

hybridomas selected during a series of subcloning procedures were used for further characterization in binding and functional assays.

Table 1A. A Typical Fusion

Fusion #	anti-CD40 titer		# of wells screened	# of ELISA+	# of cell surface+
	(1:100K)	Fusion Efficiency			
153	3	100%	960	123	33
154	4.67	15%	140	0	0
155	6	~40%	960	3	3
156	3.17	~25%	220	1	0
157	4.67	90%	960	32	6
158	4.4	90%	960	23	8
159	1.17	100%	960	108	18
160	1.78	90%	960	30	5
Total			6120	320	73

5

Table 1B. Summary of Four Sets of Fusions

# of mice	ELISA-positive hybridomas	Cell surface positive Hybridomas
31	895	260

Table 2. Summary of initial set of data with anti-CD40 IgG1 antibodies

Mother Hybridoma	Hybridoma clones	cell surface binding	Antagonist	ADCC	CDC	CMCC#	V-region DNA sequence
	131.2F5.8.5.1	+++	++	ND	ND	ND	
131.2F5	131.2F5.8.5.9	+++	+++	++	-	12047	Yes
	131.2F5.8.5.11	+++	+++	++	-	12055	Yes
	153.3C5D8D7.8.4.7.1	++	ND	ND	ND	ND	
153.3C5	153.3C5D8D7.8.4.7.8	++	ND	ND	ND	ND	
	153.3C5D8D7.8.4.7.11	+++	+++	+	ND	ND	
	153.1D2.9.1	+++	ND	ND	ND	12067	
153.1D2	153.1D2.9.8	+++	+++	++	-	12057	
	153.1D2.9.12	+++	ND	ND	ND	12068	
	158.6F3.5.1	+++	+++	++	-	12054	Yes
158.6F3	158.6F3.5.7	+++	ND	ND	ND	12061	
	158.6F3.5.10	+++	ND	ND	ND	12062	
	153.8E2D10D6.12.7	+++	ND	ND	ND	12075	
153.8E2	153.8E2D10D6.12.9	+++	ND	ND	ND	12063	
	153.8E2D10D6.12.12	+++	+++	+++	-	12056	Yes
	155.2C2E9F12.2.10.4	+++	+/-	ND	ND	12064	
155.2C2	155.2C2E9F12.2.10.5	+++	ND	ND	ND	12065	
	155.2C2E9F12.2.10.6	+/-	ND	ND	ND	12066	
	166.5E6G12.1	+++	ND	ND	ND	12069	
166.5E6	166.5E6G12.3	+++	ND	ND	ND	12070	
	166.5E6G12.4	+++	+	ND	ND	12071	
177.8C10	177.8C10B3H9	+++	++	ND	ND	ND	
	183.4B3E11.6.1.5	++	ND	ND	ND	ND	
183.4B3	183.4B3E11.6.1.9	++	ND	ND	ND	ND	
	183.4B3E11.6.1.10	+++	++	ND	ND	ND	
	183.2G5D2.8.7	+++	+/-	ND	ND	ND	
183.2G5	183.2G5D2.8.8	+++	ND	ND	ND	ND	
	183.2G5D2.8.9	+++	ND	ND	ND	ND	
	184.6C11D3.2	++	ND	ND	ND	12078	
184.6C11	184.6C11D3.3	++	ND	ND	ND	12080	
	184.6C11D3.6	+/-	+/-	ND	ND	12079	
	185.3E4F12.5.6	+++	ND	ND	ND	12072	
185.3E4	185.3E4F12.5.11	+++	ND	ND	ND	12073	
	185.3E4F12.5.12	+++	+	ND	ND	12074	
	185.1A9E9.6.1	+	ND	ND	ND	ND	
185.1A9	185.1A9E9.6.6	+++	+++	+	ND	ND	
	185.9F11E10.3B5.1	+++	ND	ND	ND	ND	
185.9F11	185.9F11E10.3B5.8	+++	ND	ND	ND	ND	
	185.9F11E10.3B5.12	+++	+++	ND	ND	ND	

Clones from 7 mother hybridomas were identified to have antagonistic activity. Based on their relative antagonistic potency and ADCC activities, two hybridoma clones were selected. Their names are: 131.2F5.8.5.9 (5.9) and 153.8E2.D10.D6.12.12 (12.12).

Clones from 7 other hybridomas were identified as having antagonistic activity (Table 2 above). Based on their relative antagonistic potency and ADCC activities, two hybridoma clones were selected for further evaluation. They are named 131.2F8.5.9 (5.9) and 153.8E2.D10.D6.12.12 (12.12). The binding profile of these two antibodies to CD40+ lymphoma cell line is shown as a flow cytometric histogram in Figure 1.

Example 2: Polynucleotide and Amino Acid Sequences of Human Anti-CD40

Antibodies

The cDNAs encoding the variable regions of the candidate antibodies were amplified by PCR, cloned, and sequenced. The amino acid sequences for the light chain and heavy chain of the 12.12 antibody are set forth in Figures 9A and 9B, respectively. See also SEQ ID NO:2 (light chain for mAb 12.12) and SEQ ID NO:4 (heavy chain for mAb 12.12). A variant of the heavy chain for mAb 12.12 is shown in Figure 9B (see also SEQ ID NO:5), which differs from SEQ ID NO:4 in having a serine residue substituted for the alanine residue at position 153 of SEQ ID NO:4. The nucleotide sequences encoding the light chain and heavy chain of the 12.12 antibody are set forth in Figures 11A and 11B, respectively. See also SEQ ID NO:1 (coding sequence for light chain for mAb 12.12) and SEQ ID NO:3 (coding sequence for heavy chain for mAb 12.12). The amino acid sequences for the light chain and heavy chain of the 5.9 antibody are set forth in Figures 10A and 10B, respectively. See also SEQ ID NO:6 (light chain for mAb 5.9) and SEQ ID NO:7 (heavy chain for mAb 5.9). A variant of the heavy chain for mAb 5.9 is shown in Figure 10B (see also SEQ ID NO:8), which differs from SEQ ID NO:7 in having a serine residue substituted for the alanine residue at position 158 of SEQ ID NO:7.

As expected for antibodies derived from independent hybridomas, there is substantial variation in the nucleotide sequences in the complementarity determining regions (CDRs). The diversity in the CDR3 region of V_H is believed to most significantly determine antibody specificity.

Example 3: Effect of 5.9 and 12.12 on the CD40/CD40L Interaction *In vitro*

The candidate antibodies 5.9 and 12.12 prevent the binding of CD40 ligand to cell surface CD40 and displace the pre-bound CD40 ligand. Antibodies 5.9 and 12.12 were tested for their ability to prevent CD40-ligand binding to CD40 on the surface of a lymphoma cell line (Ramos). Binding of both antibodies (unlabeled) prevented the subsequent binding of PE-CD40 ligand as measured by flow cytometric assays (Figure 2A). In a second set of assays the two antibodies were tested for their ability to replace CD40 ligand pre-bound to cell surface CD40. Both antibodies were effective for competing out pre-bound CD40 ligand, with 5.9 being slightly more effective than 12.12 (Figure 2B).

Example 4: 5.9 and 12.12 Bind to a Different Epitope on CD40 Than 15B8

The candidate monoclonal antibodies 5.9 and 12.12 compete with each other for binding to CD40 but not with 15B8, an IgG₂ anti-CD40 mAb (see International Publication No. WO 02/28904). Antibody competition binding studies using Biacore were designed using CM5 biosensor chips with protein A immobilized via amine coupling, which was used to capture either anti-CD40, 12.12, or 15B8. Normal association/dissociation binding curves are observed with varying concentrations of CD40-his (data not shown). For competition studies, either 12.12 or 15B8 were captured onto the protein A surface. Subsequently a CD40-his / 5.9 Fab complex (100 nM CD40:1 μM 5.9 Fab), at varying concentrations, was flowed across the modified surface. In the case of 12.12, there was no association of the complex observed, indicating 5.9 blocks binding of 12.12 to CD40-his. For 15B8, association of the Fab 5.9 complex was observed indicating 5.9 does not block binding of 15B8 to CD40 binding site. However, the off rate of the complex dramatically increased (data not shown).

It has also been determined that 15B8 and 12.12 do not compete for CD40-his binding. This experiment was set up by capturing 12.12 on the protein A biosensor chip, blocking residual protein A sites with control hIgG₁, binding CD40-his and then flowing 15B8 over the modified surface. 15B8 did bind under these conditions indicating 12.12 does not block 15B8 from binding to CD40.

Example 5: Binding Properties of Selected Hybridomas

Protein A was immobilized onto CM5 biosensor chips via amine coupling. Human anti-CD40 monoclonal antibodies, at 1.5 µg/ml, were captured onto the modified biosensor surface for 1.5 minutes at 10 µl/min. Recombinant soluble CD40-his was flowed over the biosensor surface at varying concentrations. Antibody and antigen were diluted in 0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20 (HBS-EP). Kinetic and affinity constants were determined using the Biaevaluation software with a 1:1 interaction model/global fit.

As shown in Table 3 below, there is 121-fold difference in the off rate of 5.9 and 12.12 resulting in 24-fold higher affinity for 12.12.

Table 3. Summary of binding properties of 5.9 and 12.12 anti-CD40 antibodies.

<u>Antibody</u>	<u>ka (M⁻¹ sec⁻¹))</u>	<u>kd (sec⁻¹)</u>	<u>KD (nM)</u>
Anti-CD40, 5.9	(12.35 ± 0.64) x 10 ⁵	(15.0 ± 1.3) x 10 ⁻³	12.15 ± 0.35
Anti-CD40, 12.12	(2.41 ± 0.13) x 10 ⁵	(1.24 ± 0.06) x 10 ⁻⁴	0.51 ± 0.02

Example 6: 5.9 and 12.12 are Potent Antagonists for CD40-Mediated Proliferation of Human Lymphocytes from Normal Subjects

Engagement of CD40 by CD40 ligand induces proliferation of human B cells. An antagonistic anti-CD40 antibody is expected to inhibit this proliferation. Two candidate antibodies (5.9 and 12.12) were tested for their ability to inhibit CD40 ligand-induced proliferation of PBMC from normal human subjects. Formaldehyde-fixed CHO cells transfectant-expressing CD40 ligand (CD40L) were used as a source of CD40 ligand.

Human PBMC were cultured for 4 days with the formaldehyde-fixed CHO cells

expressing CD40-ligand in the presence of varying concentrations of anti-CD40 mAb 5.9 or 12.12. The proliferation was measured by tritiated-thymidine incorporation. Cells were pulsed with tritiated-labeled thymidine at 37°C for 14-18 hours.

Both antibodies were found to be very effective for inhibiting CD40 ligand-induced proliferation of human PBMC (Table 4A, mAb 5.9, Table 4B, mAb 12.12). The experiment was performed with multiple donors of PBMC (n=12 for 5.9 and n=2 for 12.12) to ensure that the observed inhibition was not a peculiarity of cells from a single donor. Follow-up assessments with 4 additional donors of PBMC were carried out for mAb 12.12 with similar trends observed. A wide range of antibody concentrations (0.01 µg/ml to 100 µg/ml) was used in these assays. Nearly complete inhibition of CD40 ligand-induced proliferation could be achieved at 0.1 µg/ml concentration of antibodies in most cases. Antibody concentration (pM) to inhibit 50% of CD-40 ligand-induced lymphocyte proliferation (IC50) for lymphocytes from 6 donors yielded an average IC50 (pM) of 47 (SD=21; donor 1, 24; donor 2, 66; donor 3, 45; donor 4, 84; donor 5, 30; donor 6, 35), which compares favorably with the average IC50 (pM) of 49.65 shown in Table 4B. Based on the current data set, both candidate antibodies seem similar in their potency for inhibition of CD40 ligand-induced proliferation of normal PBMC.

Table 4A. Effect of mAb 5.9 on CD40L-induced PBMC proliferation.

Table 4A. Effect of MAO 5.9 on CD40L-induced PBMC proliferation.									
Exp#		PBMC alone	CHO-CD40L alone	PBMC+ CHO-CD40L	Abs Conc (µg/ml)	hulG1 CPM	% of Inhibition	5.9 CPM	% of inhibition
PBMC-010		1851	121	4436	1	5080	-26	2622	74
		1851	121	4436	0.25	5498	-43	2907	62
		1851	121	4436	0.0625	6029	-65	2619	74
		1851	121	4436	0.0156	5814	-56	1199	131
PBMC-011	Donor#1	2162	178	8222	10	13137	-84	2252	101
		2162	178	8222	1	11785	-61	1438	115
		2162	178	8222	0.1	10758	-43	1249	119
		2162	178	8222	0.01	11322	-53	4705	60
	Donor#2	2216	294	7873	10	16679	-164	2362	103
		2216	294	7873	1	14148	-117	1202	124
		2216	294	7873	0.1	12422	-85	756	133
		2216	294	7873	0.01	13870	-112	6606	24
	Donor#3	2396	241	11021	10	11641	-7	2631	100
		2396	241	11021	1	13528	-30	1450	114
		2396	241	11021	0.1	12176	-14	990	120
		2396	241	11021	0.01	11895	-10	5357	68
	Donor#4	4552	133	15301	10	22098	-64	3768	109
		4552	133	15301	1	19448	-39	2040	125
		4552	133	15301	0.1	18398	-29	1728	128
		4552	133	15301	0.01	22767	-70	9481	55
PBMC-012	777	117	6041	10	7327	-25	2150	76	
	777	117	6041	1	6212	-3	1550	87	
	777	117	6041	0.1	7006	-19	828	101	
	777	117	6041	0.01	7524	-29	1213	94	
PBMC-014	1857	73	7889	100	9399	-25	3379	76	
	1857	73	7889	20	8120	-4	3870	67	
	1857	73	7889	4	8368	-8	2552	90	
	1857	73	7889	0.8	9564	-28	1725	103	
PBMC-015	Donor#1	3203	127	10485	100	15425	-69	1497	126
		3203	127	10485	20	11497	-14	1611	124
		3203	127	10485	4	11641	-16	1359	128
		3203	127	10485	0.8	12807	-32	1490	126
	Donor#2	3680	175	15145	100	21432	-56	1792	118
		3680	175	15145	20	16998	-16	1779	118
		3680	175	15145	4	17729	-23	1965	117
		3680	175	15145	0.8	17245	-19	2217	115
	Donor#3	2734	152	19775	100	22967	-19	1664	107
		2734	152	19775	20	21224	-9	1848	106
		2734	152	19775	4	20658	-5	1534	108
		2734	152	19775	0.8	18923	5	1262	110
PBMC-016	1118	36	13531	0.1	10928	21	745	103	
	1118	36	13531	0.05	11467	17	962	102	
	1118	36	13531	0.01	11942	13	3013	85	
PBMC-017		962	75	12510	1	13597	-9	258	107

Table 4A. Effect of mAb 5.9 on CD40L-induced PBMC proliferation (cont.).

Average % inhibition of human PBMC at 100 µg/ml	-42	107
Average % inhibition of human PBMC at 10 µg/ml	-69	98
Average % inhibition of human PBMC at 1 µg/ml	-41	107
Average % inhibition of human PBMC at 0.1 µg/ml	-28	117
Average % inhibition of human PBMC at 0.01 µg/ml	-44	64
Average % inhibition of human PBMC	-35	101

% of inhibition:

$100 - (\text{CPM with Abs-PBMC alone} - \text{CHO-CD40L alone}) / (\text{CPM of PBMC} + \text{CHO-CD40L} - \text{PBMC alone} - \text{CHO-CD40L alone}) * 100\%$

Table 4B. Effect of mAb 12.12 on CD40L-induced PBMC proliferation.									
Exp#		PBMC alone	CHO-CD40L alone	PBMC+ CHO-CD40L	Abs Conc (µg/ml)	HulG1 CPM	% of inhibition	12.12 CPM	% of inhibition IC50(nM)
PBMC-025	Donor#1	4051	32	42292	0.1	33354	23	440	110
		4051	32	42292	0.01	37129	14	8696	88
		4051	32	42292	0.001	40271	5	32875	25
		4051	32	42292	0.0001	40034	6	37261	13 24.22
	Donor#2	2260	31	14987	0.1	15767	-6	365	115
		2260	31	14987	0.01	17134	-17	6734	65
		2260	31	14987	0.001	20142	-41	16183	-9
		2260	31	14987	0.0001	17847	-23	16187	-9 65.96
PBMC-026	Donor#1	2039	35	19071	0.1	17136	11	624	109
		2039	35	19071	0.01	16445	15	6455	74
		2039	35	19071	0.001	16195	17	17833	7
		2039	35	19071	0.0001	18192	5	17924	7 45
	Donor#2	2016	64	17834	0.1	17181	4	2078	100
		2016	64	17834	0.01	16757	7	10946	44
		2016	64	17834	0.001	18613	-5	17924	-1
		2016	64	17834	0.0001	17169	4	18569	-5 84
PBMC-028	Donor#1	4288	45	22547	1	18204	24	2098	112
		4288	45	22547	0.1	20679	10	1827	114
		4288	45	22547	0.01	22799	-1	6520	88
		4288	45	22547	0.001	23547	-5	22327	1
		4288	45	22547	0.0001	24778	-12	24124	-9 30.07
	Donor#2	2148	58	54894	1	48545	12	5199	94
		2148	58	54894	0.1	45708	17	5091	95
		2148	58	54894	0.01	51741	6	18890	68
		2148	58	54894	0.001	52421	5	50978	7
		2148	58	54894	0.0001	54778	0	52581	4 34.68
PBMC-029	Donor#1	609	69	10054	0.1	11027	-10	2098	85
		609	69	10054	0.01	10037	0	1827	88
		609	69	10054	0.001	10222	-2	6520	38
		609	69	10054	0.0001	11267	-13	22327	-131 28.06
	Donor#2	7737	57	23132	0.1	21254	12	2536	134
		7737	57	23132	0.01	21726	9	10249	84
		7737	57	23132	0.001	22579	4	23380	-2
		7737	57	23132	0.0001	22491	4	23183	0 55.35
PBMC-030	Donor#1	2739	47	53426	0.1	60116	-13	2132	101
		2739	47	53426	0.01	56411	-6	14297	77
		2739	47	53426	0.001	59167	-11	55868	-5
		2739	47	53426	0.0001	59290	-12	60865	-15 35.52
	Donor#2	4310	50	53781	0.1	52881	2	3208	102
		4310	50	53781	0.01	51741	4	30716	47
		4310	50	53781	0.001	53072	1	53628	0
		4310	50	53781	0.0001	58045	-9	54343	-1 102.88
PBMC-032	Donor#1	2458	42	14058	0.1	16579	-22	636	116 40.36
		2458	42	14058	0.01	19250	-45	3358	93

2458	42	14058	0.001	19852	-50	20639	-57
2458	42	14058	0.0001	19161	-44	18907	-42
Average % inhibition of human PBMC at 0.1 µg/ml					3		107
Average % inhibition of human PBMC at 0.01 µg/ml					-1		74
Average % inhibition of human PBMC at 0.001 µg/ml					-7		0
Average % inhibition of human PBMC at 0.0001 µg/ml					-8		-17 49.65
% of inhibition: 100-(CPM with Abs-PBMC alone-CHO-CD40L alone)/(CPM of PBMC+CHO-CD40L-PBMC alone-CHO-CD40L alone)*100%							

In addition to B cells, human PBMC also contain natural killer cells that can mediate antibody dependent cytotoxicity (ADCC). To clarify the mechanism of antibody-mediated inhibition of proliferation, assays were performed with B cells purified from human PBMC. Similar to results obtained with PBMC, both antibodies potently inhibited the CD40 ligand-induced proliferation of purified B cells (Table 5, n=3). These data demonstrate that the antagonistic activity of the candidate antibodies, and not the mechanism of ADCC, is the cause of proliferation inhibition in these assays.

Table 5. Effect of anti-CD40 antibodies on CD40 ligand-induced proliferation of purified human B cells

Exp#	Donor #	CPM			Abs Conc(µg/ml)	HuIgG1		5.9		12.12	
		B cells	CHO-CD40L	B cells + CHO-CD40L		CPM	% inhibition	CPM	% inhibition	CPM	% inhibition
B cell-004	1	418	89	3132	100	429	103	271	109	152	114
		418	89	3132	20	3193	-2	316	107	222	111
		418	89	3132	4	3175	-2	144	114	235	110
		418	89	3132	0.8	6334	-122	245	110	63	117
	2	81	73	27240	100	28311	-4	85	100	77	100
		81	73	27240	20	24707	9	65	100	94	100
		81	73	27240	4	23081	15	108	100	68	100
		81	73	27240	0.8	26252	4	87	100	77	100
B cell-005	3	267	75	24552	1	25910	-6	291	100	102	101
		267	75	24552	0.1	28447	-16	259	100	108	101
		267	75	24552	0.01	26706	-9	2957	89	4922	81
Average % inhibition							-3	103	103		

% of inhibition: 100-(CPM with Abs-B cells alone-CHO-CD40L alone)/(CPM of B cell with CHO-CD40L-B cells alone-CHO-CD40L alone)*100%

Example 7: 5.9 and 12.12 Do Not Induce Strong Proliferation of Human B Cells from Normal Subjects

CD40 ligand activates normal B cells and B-cell lymphoma cells to proliferate. Binding of some anti-CD40 antibodies (agonist) can provide a similar stimulatory signal for the proliferation of normal and cancer B cells. Antibodies with strong B cell

stimulatory activity are not suitable candidates for therapeutic treatment of B cell lymphomas. The two candidate antibodies were tested for their ability to induce proliferation of B cells from normal volunteer donors. The B cells purified by Ficoll-Hypaque Plus gradient centrifugation from normal donor PBMC were cultured in 96-well plates with varying concentrations of candidate antibodies (range of 0.001 to 100 µg/ml) for a total of 4 days. In the positive control group, PBMC were cultured with formaldehyde-fixed CHO cells expressing CD40-ligand. The B cell proliferation was measured by incorporation of tritiated-labeled thymidine at 37°C for 14-18 hours. While the CD40 ligand presented on CHO cells induced vigorous proliferation of B cells resulting in an average stimulation index (SI) of 145, the candidate antibodies induced only a weak proliferation with a stimulation index of 2.89 and 5.08 for 12.12 and 5.9, respectively (n=3) (Table 6).

Table 6. Proliferation of B cells purified from normal human subjects in response to candidate anti-CD40 mAbs

anti-CD40 mAbs												
Exp#	Donor#	B cells		Bcells+CHO-CD40L		Abs conc (ug/ml)	B cells+hulIgG1		B cells+5.9		B cells+12.12	
		CPM	CPM	S. index(1)	CPM		S. index(2)	CPM	S. index (2)	CPM	S. index(2)	
B cell-004 Frozen	1	418	3132	7.49	100	498	1.19	401	0.96	458	1.10	
		418	3132		20	245	0.59	232	0.56	370	0.89	
		418	3132		4	241	0.58	232	0.56	211	0.50	
		418	3132		0.8	376	0.90	298	0.71	230	0.55	
Frozen	2	81	27240	336.30	100	34	0.42	454	5.60	122	1.51	
		81	27240		20	48	0.59	706	8.72	255	3.15	
		81	27240		4	41	0.51	567	7.00	367	4.53	
		81	27240		0.8	34	0.42	736	9.09	408	5.04	
B cell-005	3	267	24552	91.96	1	691	2.59	2101	7.87	1223	4.58	
		267	24552		0.1	686	2.57	2267	8.49	1557	5.83	
		267	24552		0.01	808	3.03	2203	8.25	1027	3.85	
		267	24552		0.001	567	2.12	846	3.17	826	3.09	
Average Stimulation Index (SI)				145.25			1.29	5.08		2.88		
S. index(1): = CPM (B cells+CHO-CD40L)/CPM (B cells alone)												
S. index(2): = CPM (B cells+Abs)/CPM (PBMC alone)												

In addition to B cells, human PBMC contain cell types that bear Fc receptors (FcR) for IgG1 molecules that can provide cross linking of anti-CD40 antibodies bound to CD40 on B cells. This cross-linking could potentially enhance stimulatory activity of anti-CD40 antibodies. To confirm the lack of B cell stimulatory activity of 5.9 and 12.12 antibodies in the presence of cross-linking cells, proliferation experiments were

performed with total PBMC containing B cells as well as FcR+ cells. Data from these experiments (Table 7A, mAb 5.9; Table 7B, mAb 12.12) confirm that these candidate antibodies even in the presence of FcR-bearing cells in general do not stimulate B cells to proliferate over background proliferation induced by control human IgG1 (n=10). The CD40 ligand induced an average stimulation index (SI) SI of 7.41. The average SI with candidate antibodies were 0.55 and 1.05 for 12.12 and 5.9, respectively. Only one of the 10 donor PBMC tested showed some stimulatory response to 5.9 antibody (donor #2 in Table 7). The lack of stimulatory activity by candidate mAbs was further confirmed by measuring the PBMC proliferation in response to candidate anti-CD40 antibodies immobilized on the plastic

Table 7A. Proliferation of PBMC from normal human subjects in response to mAb 5.9.

Exp#		PBMC+CHO-CD40L			Abs conc (μ g/ml)	PBMC+hulG1		PBMC+5.9	
		PBMC	CPM	Index(1)		CPM	Index(2)	CPM	Index(2)
PBMC-010		1417	5279	3.73	1	1218	0.86	5973	4.22
		1417	5279		0.25	1712	1.21	4815	3.40
		1417	5279		0.0625	1449	1.02	3642	2.57
		1417	5279		0.0156	1194	0.84	3242	2.29
PBMC-011 Donor#1		2138	8247	3.86	10	3047	1.43	3177	1.49
		2138	8247		1	2726	1.28	3617	1.69
		2138	8247		0.1	2026	0.95	2011	0.94
		2138	8247		0.01	2424	1.13	1860	0.87
	Donor#2	2374	11561	4.87	10	4966	2.09	4523	1.91
		2374	11561		1	2544	1.07	2445	1.03
		2374	11561		0.1	2177	0.92	1462	0.62
		2374	11561		0.01	4672	1.97	1896	0.80
	Donor#3	3229	7956	2.46	10	5035	1.56	2119	0.66
		3229	7956		1	2826	0.88	1099	0.34
		3229	7956		0.1	2277	0.71	1052	0.33
		3229	7956		0.01	3078	0.95	1899	0.59
	Donor#4	4198	14314	3.41	10	5012	1.19	5176	1.23
		4198	14314		1	3592	0.86	4702	1.12
		4198	14314		0.1	5298	1.26	4319	1.03
		4198	14314		0.01	5758	1.37	5400	1.29
PBMC-014		2350	8787	3.74	100	2722	1.16	2471	1.05
		2350	8787		20	2315	0.99	2447	1.04
		2350	8787		4	2160	0.92	1659	0.71
		2350	8787		0.8	2328	0.99	1671	0.71
PBMC-015 Donor#1		3284	12936	3.94	100	3598	1.10	1682	0.51
		3284	12936		20	2751	0.84	1562	0.48
		3284	12936		4	3135	0.95	1105	0.34
		3284	12936		0.8	4027	1.23	1419	0.43
	Donor#2	6099	19121	3.14	100	2999	0.49	5104	0.84
		6099	19121		20	4025	0.66	3917	0.64
		6099	19121		4	4496	0.74	3341	0.55
		6099	19121		0.8	3834	0.63	4139	0.68
	Donor#3	2479	19826	8.00	100	3564	1.44	1204	0.49
		2479	19826		20	1874	0.76	782	0.32
		2479	19826		4	1779	0.72	634	0.26
		2479	19826		0.8	2274	0.92	937	0.38
PBMC-016		1148	15789	13.75	0.1	1255	1.09	1036	0.90
		1148	15789		0.05	1284	1.12	871	0.76
		1148	15789		0.01	1446	1.26	952	0.83
Average SI of PBMC				5.09			1.06		1.03

Index(1): =(PBMC+CHO-CD40L)/PBMC

Index(2): =(PBMC+Abs)/PBMC

Table 7B. Proliferation of PBMC from normal human subjects in response to mAb 12.12.

Exp#		PBMC	PBMC+CHO-CD40L		Abs conc (μ g/ml)	PBMC+hulG1		PBMC+12.12	
			CPM	Index(1)		CPM	Index(2)	CPM	Index(2)
PBMC-025	Donor#1	4051	42292	10.44	0.1	2909	0.72	2451	0.61
		4051	42292		0.01	4725	1.17	8924	2.20
		4051	42292		0.001	8080	1.99	8782	2.17
		4051	42292		0.0001	4351	1.07	4342	1.07
	Donor#2	2260	14987	6.63	0.1	2538	1.12	6741	2.98
		2260	14987		0.01	3524	1.56	8921	3.95
		2260	14987		0.001	3159	1.40	4484	1.98
		2260	14987		0.0001	2801	1.24	2533	1.12
PBMC-026	Donor#1	2085	18313	8.78	0.1	1386	0.66	2761	1.32
		2085	18313		0.01	2871	1.38	3162	1.52
		2085	18313		0.001	2602	1.25	3233	1.55
		2085	18313		0.0001	1709	0.82	1766	0.85
	Donor#2	676	18054	26.71	0.1	660	0.98	2229	3.30
		676	18054		0.01	2864	4.24	1238	1.83
		676	18054		0.001	693	1.03	1507	2.23
		676	18054		0.0001	984	1.46	811	1.20
PBMC-027	Donor#1	2742	13028	4.75	0.1	4725	1.72	2795	1.02
		2742	13028		0.01	4575	1.67	5353	1.95
		2742	13028		0.001	3218	1.17	3501	1.28
		2742	13028		0.0001	5107	1.86	4272	1.56
	Donor#2	1338	11901	8.89	0.1	1633	1.22	1943	1.45
		1338	11901		0.01	1520	1.14	5132	3.84
		1338	11901		0.001	1517	1.13	2067	1.54
		1338	11901		0.0001	1047	0.78	2076	1.55
PBMC-028	Donor#1	4288	22547	5.26	0.1	3686	0.86	2525	0.59
		4288	22547		0.01	3113	0.73	2047	0.48
		4288	22547		0.001	4414	1.03	3515	0.82
		4288	22547		0.0001	2452	0.57	4189	0.98
	Donor#2	2148	54894	25.56	0.1	9127	4.25	5574	2.59
		2148	54894		0.01	4566	2.13	6515	3.03
		2148	54894		0.001	5285	2.46	5919	2.76
		2148	54894		0.0001	4667	2.17	4298	2.00
PBMC-029	Donor#1	609	10054	16.51	0.1	359	0.59	363	0.60
		609	10054		0.01	473	0.78	956	1.57
		609	10054		0.001	461	0.76	1159	1.90
		609	10054		0.0001	625	1.03	558	0.92
	Donor#2	7737	23132	2.99	0.1	4940	0.64	3493	0.45
		7737	23132		0.01	6041	0.78	3644	0.47
		7737	23132		0.001	5098	0.66	5232	0.68
		7737	23132		0.0001	5135	0.66	5241	0.68
PBMC-030	Donor#1	4164	57205	13.74	10	2713	0.65	1046	0.25
		4164	57205		1	3627	0.87	1576	0.38
		4164	57205		0.1	4590	1.10	1512	0.36
		4164	57205		0.01	4384	1.05	2711	0.65
	Donor#2	3324	53865	16.20	10	6376	1.92	4731	1.42
		3324	53865		1	4720	1.42	5219	1.57
		3324	53865		0.1	3880	1.17	5869	1.77
		3324	53865		0.01	3863	1.16	5657	1.70

PBMC-032	Donor#1	1808	15271	8.45	10	2349	1.30	4790	2.65
		1808	15271		1	3820	2.11	5203	2.88
		1808	15271		0.1	2098	1.16	6332	3.50
		1808	15271		0.01	1789	0.99	5005	2.77
Average SI of PBMC				11.92			1.30	1.62	

index(1): =CPM of (PBMC+CHO-CD40L)/CPM of PBMC

index(2): =CPM of (PBMC+Abs)/CPM of PBMC

5 surface of the culture wells (n=2). The average SI with CD40 ligand, 12.12, and 5.9 stimulation were 22, 0.67, and 1.2, respectively (Table 8). Taken together these data show that the candidate antiCD40 antibodies do not possess strong B cell stimulatory properties.

Table 8. Proliferation of PBMC from normal human subjects in response to immobilized anti-CD40 antibodies

Exp#	PBMC	PBMC+CHO-CD40L		Abs conc (ug/ml)	PBMC+huIgG1		PBMC+5.9		PBMC+12.12	
	CPM	CPM	S.index (1)		CPM	S. index(2)	CPM	S.index (2)	CPM	S.index (2)
PBMC-012	225	6808	30.26	10	279	1.24	734	3.26	200	0.89
	225	6808		1	175	0.78	178	0.79	161	0.72
	225	6808		0.1	156	0.69	226	1.00	249	1.11
	225	6808		0.01	293	1.30	232	1.03	254	1.13
Immoblize-004	857	11701	13.65	1000	479	0.56	1428	1.67	384	0.45
	857	11701		100	543	0.63	839	0.98	265	0.31
	857	11701		10	487	0.57	411	0.48	262	0.31
	857	11701		1	632	0.74	372	0.43	376	0.44

Average Stimulation index 21.96 0.81 1.21 0.67

S. index (1): = CPM (PBMC+CHO-CD40L)/CPM (PBMC)

S. index (2): = CPM (PBMC+mAbs)/CPM (PBMC)

Example 8: 5.9 and 12.12 Are Able to Kill CD40-Bearing Target Cells by ADCC

The candidate antibodies can kill CD40-bearing target cells (lymphoma lines) by the mechanism of ADCC. Both 5.9 and 12.12 are fully human antibodies of IgG1 isotype and are expected to have the ability to induce the killing of target cells by the mechanism of ADCC. They were tested for their ability to kill cancer cell lines in *in vitro* assays. Two human lymphoma cell lines (Ramos and Daudi) were selected as target cells for these assays. PBMC or enriched NK cells from 8 normal volunteer donors were used as effector cells in these assays. A more potent ADCC response was observed with 12.12 compared with 5.9 against both the lymphoma cancer cell line target cells. Lymphoma cell lines also express CD20, the target antigen for rituximab (Rituxan®), which allowed

for comparison of the ADCC activity of these two candidate mAbs with rituximab ADCC activity. For lymphoma cell line target, an average specific lysis of 35%, 59%, and 47% was observed for 5.9, 12.12, and rituximab respectively when used at 1 µg/ml concentration (Table 9). The two antibodies did not show much activity in complement dependent cytotoxicity (CDC) assays.

Table 9. Anti-CD40 mAB dependent killing of lymphoma cell lines by ADCC.

Anti-CD40 mAb dependent killing of lymphoma and colon cancer cell lines by ADCC										
Exp#	Effector cell	E:T ratio	Target cells : Human lymphoma cell line (Ramos or Daudi)							
			% lysis IgG1	Abs conc(µg/ml)	5.9		12.12		Rituxan	
					% lysis	% lysis - % lysis IgG1	% lysis	% lysis-% lysis IgG1	% lysis	% lysis-% lysis IgG1
ADCC-005	huNK	3	17.05	5	30.75	13.70	65.22	48.17	ND	ND
Alarmor Blue	huNK	3	40.81	5	58.62	17.81	87.87	47.06	ND	ND
ADCC-006	huNK	2	-3.09	10	3.50	6.59	43.71	46.8	34.82	37.91
Alarmor Blue			-8.62	1	-10.10	-1.48	45.13	53.75	37.07	45.69
			-11	0.1	-14.80	-3.80	39.82	50.82	33.61	44.61
			-4.54	0.01	2.53	7.07	50.07	54.61	28.49	33.03
51Cr	huNK	5	1.5	10	32.09	30.59	47.24	45.742	ND	ND
			2.4	1	18.01	15.61	37.42	35.022	ND	ND
			2.5	0.1	14.67	12.17	37.63	35.131	ND	ND
ADCC-009	huNK	10	2.32	5	66.20	63.88	97.70	95.38	86.2	83.88
Calcein AM			0.48	1	67.20	66.72	123.00	122.52	88.2	87.72
			-1.43	0.2	78.40	79.83	118.00	119.43	88.8	90.23
			3.39	0.04	69.10	65.71	109.00	105.61	84.9	81.51
ADCC-011	huNK	8	3.18	1	15.36	12.19	51.59	48.42	22.44	19.27
Calcein AM			4.58	0.01	7.39	2.81	46.80	42.22	14.68	10.10
			5.41	0.002	6.35	0.94	5.10	-0.31	9.58	4.16
			7.03	0.0004	7.76	0.73	5.99	-1.04	5.99	-1.04
ADCC-012	huNK	10	13.34	10	73.31	59.97	117.80	104.46	50.75	37.41
Calcein AM			13.50	1	74.76	61.26	88.64	75.14	65.97	52.47
			12.27	0.01	58.52	46.25	72.88	60.61	50.16	37.89
			13.61	0.005	57.50	43.89	69.45	55.84	39.28	25.67
			11.95	0.001	56.81	44.86	65.17	53.22	33.07	21.12
ADCC-013	PBMC	100	2.54	1	21.03	18.49	37.94	35.40	32.28	29.74
51Cr			2.45	0.1	15.50	13.05	30.82	28.37	27.18	24.73
			2.92	0.01	14.53	11.61	22.59	19.67	12.79	9.87
			2.78	0.001	3.90	1.12	8.99	6.21	3.13	0.35
ADCC-014	PBMC	100	4.64	10	53.54	48.90	56.12	51.48	ND	ND
51Cr			4.64	1	46.84	42.20	43.00	38.36	ND	ND
			4.64	0.1	45.63	40.99	39.94	35.30	ND	ND
			4.64	0.01	7.73	3.09	9.79	5.15	ND	ND
			4.64	0.001	8.83	4.19	10.81	6.17	ND	ND
Average % lysis at 1 µg/ml concentration of mAbs						35.31		59.03		47.23
* The greater than 100% killing are due to incomplete killing by detergent used for 100% killing control.										

Example 9: CD40 Is Present on the Surface of NHL Cells from Lymph Node Biopsy

Patients

NHL cells were isolated from biopsied lymph nodes from patients and were
5 preserved in liquid nitrogen until use. Cell viability at the time of analysis exceeded
90%. The cells from two rituximab-sensitive and three rituximab-resistant patients (five
patients in total) were stained with either a direct labeled 15B8-FITC or 15B8 plus anti-
huIgG₂-FITC and analyzed by Flow cytometry. NHL cells from all the patients were
found to express CD40. Table 10 shows that an average of 76% of NHL cells express
10 CD40 (a range of 60-91%).

Table 10.

Patient ID ^a	Patient type ^b	% positive ^c	
		MS81 ^d	15B8 ^e
B	CR	n.d. ^f	91.02
J	CR	n.d.	60.36
H	NR	n.d.	85.08
H	NR	72.24	81.19
K	NR	n.d.	70.69
L	NR	n.d.	66.82

Average % positive

76

^a NHL patients treated with anti-CD20 mAb

^b patient response to anti- CD20 mAb; CR=complete responder; NR=nonresponder

^c % of cells in lymphocyte gate that stain positive

^d MS81, agonist anti-CD40 mAb

^e 15B8, antagonist anti-CD40 Mab

^f n.d., not done

Example 10: 5.9 and 12.12 Do Not Stimulate Proliferation of Cancer Cells from the
Lymph Nodes of NHL Patients

CD40 ligand is known to provide a stimulatory signal for the survival and
proliferation of lymphoma cells from NHL patients. Binding of some anti-CD40
25 antibodies (agonist) can provide a similar stimulatory signal for the proliferation of
patient cancer cells. Antibodies with strong B cell stimulatory activity are not suitable
candidate for therapeutic treatment of B cell lymphomas. The two candidate antibodies
were tested for their ability to induce proliferation of NHL cells from 3 patients. The cells

isolated from lymph node (LN) biopsies were cultured with varying concentrations of candidate antibodies (range of 0.01 to 300 µg/ml) for a total of 3 days. The cell proliferation was measured by incorporation of tritiated thymidine. Neither of the two candidate mAbs induced any proliferation of cancer cells at any concentration tested (Table 11). Antibodies even in the presence of exogenously added IL-4, a B cell growth factor, did not induce proliferation of NHL cells (tested in one of the three patient cells. These results indicate that 5.9 and 12.12 are not agonist anti-CD40 antibodies and do not stimulate proliferation *in vitro* of NHL cells from patients.

Table 11. Proliferation of cancer cells from LN of NHL patients in response to candidate anti-CD40 mAbs

Donor#	Abs conc(ug/ml)	CPM		S. index	CPM	S. index	CPM	S. index
		Cells + IgG1	Cells +5.9	5.9	cells + 12.12	12.12	cells +MS81	MS81
PP	0.01	180	203	1.23	133.67	0.74	ND	ND
	0.1	107.5	151.67	1.41	136	1.27	ND	ND
	1	130.67	206.67	1.58	197.33	1.51	179	1.37
	10	152.5	245	1.61	137.33	0.90	871.67	5.71
	100	137.67	332.33	2.41	157.33	1.14	ND	ND
	300	137.67	254.33	1.85	100.67	0.73	ND	ND
MM	0.01	165	180.33	1.09	124	0.75	ND	ND
	0.1	180.5	149.67	0.83	111.33	0.62	ND	ND
	1	62	109.67	1.77	104.67	1.69	ND	ND
	10	91.5	93.33	1.02	100	1.09	763	8.34
	100	123	173	1.41	105.33	0.86	ND	ND
	300	109	183.67	1.69	157	1.44	ND	ND
BD (IL-4)	0.01	1591.5	1623.67	1.02	1422	0.89	ND	ND
	0.1	1405	1281	0.91	1316.33	0.94	ND	ND
	1	1526	1352.33	0.89	1160	0.76	1508.33	0.99
	10	1450	1424	0.98	1244	0.86	4146.67	2.86
	100	1406.67	1497.67	1.06	1255.33	0.89	ND	ND
	300	1410.33	1466.67	1.04	1233	0.87	ND	ND

10

Example 11: 5.9 and 12.12 Can Block CD40 Ligand-Mediated Proliferation of Cancer Cells from Non-Hodgkin's Lymphoma Patients

Engagement of CD40 by CD40 ligand induces proliferation of cancer cells from NHL patients. An antagonistic anti-CD40 antibody is expected to inhibit this

proliferation. The two candidate anti-CD40 antibodies were tested at varying concentrations (0.01 $\mu\text{g/ml}$ to 100 $\mu\text{g/ml}$) for their ability to inhibit CD40 ligand-induced proliferation of NHL cells from patients. NHL cells from patients were cultured in suspension over CD40L-expressing feeder in the presence of IL-4. The NHL cell proliferation was measured by ^3H -thymidine incorporation. Both antibodies were found to be very effective for inhibiting CD40 ligand-induced proliferation of NHL cells (Table 12, n=2). Nearly complete inhibition of CD40 ligand-induced proliferation could be achieved at 1.0 to 10 $\mu\text{g/ml}$ concentration of antibodies.

Table 12. Inhibition of CD40 ligand-induced proliferation of cancer cells from the LN of NHL patients.

Patient	Abs Conc ($\mu\text{g/ml}$)	CPM IgG1	5.9		12.12		Rituximab	
			CPM	% inhibition	CPM	% inhibition	CPM	% inhibition
BD	0.01	29525.5	25369	14	24793	16	29490.3	0
	0.1	29554	20265.33	31	13671	54	29832.7	-1
	1	29486.67	6785.33	77	453	98	26355.3	11
	10	29710	506.33	98	371	99	29427.3	1
	100	29372.33	512.33	98	386.67	99	ND	ND
PP	0.01	23572	23229.33	1	23666	0	25317.3	-7
	0.1	22520	19092.33	15	17197	24	26349.7	-17
	1	23535.67	1442.33	94	802.67	97	26515.7	-13
	10	23101.5	608.67	97	221.33	99	25478.3	-10
	100	23847.33	ND	ND	252	99	ND	ND

% inhibition: $100 - (\text{CPM with test Abs} / \text{CPM with control mAb}) * 100\%$

Example 12: Effect of 5.9 on Number of Viable NHL Cells When Cultured with CD40-Ligand Bearing Cells

Effects of 5.9 on the viable NHL cell numbers when cultured with CD40-ligand bearing cells over an extended period of time (days 7, 10, and 14) were investigated. CD40 ligand-mediated signaling through CD40 is important for B cell survival. This set of experiments evaluated the effect of anti-CD40 antibodies on NHL cell numbers at days 7, 10, and 14. NHL cells from five patients were cultured in suspension over CD40L-expressing irradiated feeder cells in the presence of IL-4. The control human IgG and 5.9 antibodies were added at concentrations of 10 $\mu\text{g/ml}$ at day 0 and day 7. The viable cells under each condition were counted on the specified day. Cell numbers in the control

group (IgG) increased with time as expected. Reduced numbers of cells were recovered from 5.9-treated cultures compared to control group. The greatest levels of reduction in cell numbers by 5.9 antibody were observed at day 14 and were on average 80.5% (a range of 49-94%) compared to isotype control (n=5). These data are summarized in Table

5 13.

Table 13. Effect of anti-CD40 antibody (5.9/5.11) on NHL patient cell numbers over prolonged culture period (day 7, 10, and 14)

Patient	Days in culture	Viable cell numbers		% reduction compared to IgG control
		IgG	mAb 5.9/5.11	
PS	0	1000000	1000000	0.00
	7	935000	447500	52.14
	10	1270900	504100	60.34
	14	1029100	525000	48.98
MT	0	1000000	1000000	0.00
	7	267600	182500	31.80
	10	683400	191600	71.96
	14	1450000	225000	84.48
BRF	0	250000	250000	0.00
	7	145000	86667	40.23
	10	207500	65000	68.67
	14	570500	33330	94.16
DP	0	250000	250000	0.00
	7	188330	136670	27.43
	10	235000	128330	45.39
	14	428330	58330	86.38
PP	0	250000	250000	0.00
	7	270000	176670	34.57
	10	311670	128330	58.83
	14	458330	53330	88.36

* % reduction compare to ctrl Abs=100-(test Abs/ctrl Abs)*100

10 Example 13: 5.9 and 12.12 Are Able to Kill Cancer Cells from the Lymph Nodes of NHL Patients by ADCC

Both 5.9 and 12.12 are fully human antibodies of IgG₁ isotype and were shown to induce the killing of lymphoma cell lines *in vitro* by the mechanism of ADCC (Table 9). They were tested for their ability to kill cancer cells from a single NHL patient in *in vitro* assays. Enriched NK cells from normal volunteer donor either fresh after isolation or

after culturing overnight at 37°C were used as effector cells in this assay. Similar results were obtained with both freshly isolated NK cells and NK cells used after overnight culture. The higher level of ADCC was observed with 12.12 compared with 5.9 against the NHL cells from the patient. NHL cells also express CD20, the target antigen for rituximab (Rituxan®), which allowed for comparison of the ADCC activity of these two candidate mAbs with rituximab. Antibody 12.12 and rituximab show similar level of ADCC activity with 5.9 scoring lower in this assay. These data are shown in Figures 3A and 3B.

10 Example 14: 5.9 and 12.12 Can Block CD40-Mediated Survival and Proliferation of Cancer Cells from CLL Patients

The candidate antibodies can block CD40-mediated survival and proliferation of cancer cells from CLL patients. CLL cells from patients were cultured in suspension over CD40L-expressing formaldehyde-fixed CHO cells under two different conditions: addition of human isotype antibody IgG (control); and addition of either 5.9 or 12.12 monoclonal antibody. All antibodies were added at concentrations of 1, 10, and 100 µg/mL in the absence of IL-4. The cell counts were performed at 24 and 48 h by MTS assay. Reduced numbers of cells were recovered from 5.9- (n=6) and 12.12- (n=2) treated cultures compared to control group. The greater differences in cell numbers between anti-CD40 mAb-treated and control antibody-treated cultures were seen at the 48-h time point. These data are summarized in Table 14.

Table 14. The effect of candidate antibodies on CD40-induced survival and proliferation of cancer cells from CLL patients measured at 48 h after the culture initiation

Patient#	Ab conc(μ g/ml)	Relative cell numbers			% reduction in cell numbers*	
		IgG1	5.9/5.11	12.12	5.9/5.11	12.12
1	1	269.31	25.27	ND	90.62	ND
	10	101.58	33.07	ND	67.44	ND
	100	130.71	40.16	ND	69.28	ND
2	1	265.55	75.8	ND	71.46	ND
	10	227.57	128.5	ND	43.53	ND
	100	265.99	6.4	ND	97.59	ND
3	1	85.9	35.39	ND	58.80	ND
	10	70.44	39.51	ND	43.91	ND
	100	77.65	20.95	ND	73.02	ND
4	1	80.48	15.03	ND	81.32	ND
	10	63.01	19.51	ND	69.04	ND
	100	55.69	3.65	ND	93.45	ND
5	1	90.63	91.66	89.59	-1.14	1.15
	10	78.13	82.28	60.41	-5.31	22.68
	100	63.53	86.47	39.59	-36.11	37.68
6	1	130.21	77.6	71.88	40.40	44.80
	10	131.77	78.13	73.96	40.71	43.87
	100	127.08	76.56	82.29	39.75	35.25

* % reduction compared to control Abs=100-(test Abs/control Abs)*100

5

Example 15: 5.9 and 12.12 Show Anti-Tumor Activity in Animal Models

Pharmacology/in vivo efficacy

The candidate mAbs are expected to produce desired pharmacological effects to reduce tumor burden by either/both of two anti-tumor mechanisms, blockade of proliferation/survival signal and induction of ADCC. The currently available xenograft human lymphoma models use long-term lymphoma cell lines that, in contrast to primary cancer cells, do not depend on CD40 stimulation for their growth and survival. Therefore the component of these mAbs' anti-tumor activity based on blocking the tumor proliferation/survival signal is not expected to contribute to anti-tumor efficacy in these models. The efficacy in these models is dependent on the ADCC, the second anti-tumor mechanism associated with the 5.9 and 12.12 mAbs. Two xenograft human lymphoma models based on Namalwa and Daudi cell lines were assessed for anti-tumor activities of

candidate mAbs. To further demonstrate their therapeutic activity, these candidate mAbs were evaluated in an unstaged and staged xenograft human lymphoma model based on the Daudi cell line.

5 *Summary of in vivo efficacy data*

When administered intraperitoneally (i.p.) once a week for a total of 3 doses, 12.12, one of the two candidate mAbs, significantly inhibited the growth of aggressive unstaged B cell lymphoma (Namalwa) in a dose-dependent manner (Figure 4). The second candidate mAb, 5.9, was tested only at a single dose in this study and was less effective than 12.12 at the same dose. Interestingly, 12.12 was found to be more efficacious in this model than rituximab. It is possible that lower efficacy by rituximab could be due to low CD20 expression on the Namalwa lymphoma cell line. The efficacy observed with candidate mAbs has greater importance because only one of the two cancer cell killing mechanisms (ADCC) is operative in the current xenograft lymphoma model. Two killing mechanisms, ADCC and blocking of survival signal, are expected to contribute to anti-tumor activities in human lymphoma patients. This is likely to increase the chance of achieving efficacy in human lymphoma patients. The candidate anti-CD40 mAbs also showed a trend toward tumor growth inhibition in a second B cell lymphoma model (non-validated Daudi model, data not shown). In follow-up studies, the two candidate antibodies were shown to have dose-dependent anti-tumor efficacy in both the unstaged and staged Daudi lymphoma models (Figures 5 and 6, respectively). In the staged Daudi model, the 12.12 mAb was more efficacious at reducing tumor volume than was a similar dose of Rituxan®.

25 *Xenograft human B cell Lymphoma models*

To ensure consistent tumor growth, T cell-deficient nude mice were whole-body irradiated at 3 Gy to further suppress the immune system one day before tumor inoculation. Tumor cells were inoculated subcutaneously in the right flank at 5×10^6 cells per mouse. Treatment was initiated either one day after tumor implantation (unstaged subcutaneous xenograft human B cell lymphoma models, Namalwa and Daudi) or when tumor volume reached 200-400 mm³ (staged Daudi model, usually 15 days after tumor

inoculation). Tumor-bearing mice were injected anti-CD40 mAbs intraperitoneally (i.p.) once a week at the indicated doses. Tumor volumes were recorded twice a week. When tumor volume in any group reached 2500 mm³, the study was terminated. Note that in the staged Daudi model, tumor volume data was analyzed up to day 36 due to the death of some mice after that day. Complete regression (CR) was counted until the end of the study. Data were analyzed using ANOVA or Kruskal-Wallis test and corresponding post-test for multi-group comparison.

In the unstaged Namalwa model, anti-CD40 mAb 12.12, but not Rituxan® (rituximab), significantly ($p < 0.01$) inhibited the growth of Namalwa tumors (tumor volume reduction of 60% versus 25% for rituximab, $n=10$ mice/group) (Figure 4). Thus, in this model, anti-CD40 mAb 12.12 was more potent than rituximab. It is noteworthy that the second candidate mAb, 5.9, was at least as efficacious as rituximab at a dose 1/10th that of rituximab. Both anti-CD40 mAb 12.12 and rituxan significantly prevented tumor development in the unstaged Daudi tumor model (14/15 resistance to tumor challenge) (Figure 5).

When these anti-CD40 monoclonal antibodies were further compared in a staged xenograft Daudi model, in which treatment started when the subcutaneous tumor was palpable, anti-CD40 mAb 12.12 at 1 mg/kg caused significant tumor reduction ($p=0.003$) with 60% complete regression (6/10), while rituximab at the same dose did not significantly inhibit the tumor growth nor did it cause complete regression (0/10). See Figure 6.

In summary, the anti-CD40 mAb 12.12 significantly inhibited tumor growth in experimental lymphoma models. At the same dose and regimen, mAb 12.12 showed better anti-cancer activity than did Rituxan® (rituximab). Further, no clinical sign of toxicity was observed at this dose and regimen. These data suggest that the anti-CD40 mAb 12.12 has potent anti-human lymphoma activity *in vitro* and in xenograft models and could be clinically effective for the treatment of lymphoma.

Example 16: Pharmacokinetics of 5.9 and 12.12

The pharmacokinetics of anti-CD40 mAb in mice was studied after single IV and IP dose administration. Anti-CD40 mAb exhibited high systemic bioavailability after IP

administration, and prolonged terminal half-life (>5 days) (data not shown). This pilot study was conducted to aid in the design of pharmacology studies; however, it is of little to no importance for the development activity of this mAb since this fully human mAb does not cross react with mouse CD40.

5

Example 17: Characterization of Epitope for Monoclonal Antibodies 12.12 and 5.9

To determine the location of the epitope on CD40 recognized by monoclonal antibodies 12.12 and 5.9, SDS-PAGE and Western blot analysis were performed. Purified CD40 (0.5 μ g) was separated on a 4-12% NUPAGE gel under reducing and non-reducing conditions, transferred to PVDF membranes, and probed with monoclonal antibodies at 10 μ g/ml concentration. Blots were probed with alkaline phosphatase conjugated anti-human IgG and developed using the Western Blue^R stabilized substrate for alkaline phosphatase (Promega).

10 Results indicate that anti-CD40 monoclonal antibody 12.12 recognizes epitopes on both the non-reduced and reduced forms of CD40, with the non-reduced form of CD40 exhibiting greater intensity than the reduced form of CD40 (Table 15; blots not shown). The fact that recognition was positive for both forms of CD40 indicates that this antibody interacts with a conformational epitope part of which is a linear sequence. Monoclonal antibody 5.9 primarily recognizes the non-reduced form of CD40 suggesting
20 that this antibody interacts with a primarily conformational epitope (Table 15; blots not shown).

Table 15. Domain identification.

	Domain 1	Domain 2	Domain 3	Domain 4
mAb 12.12	-	+	-	-
mAb 5.9	-	+	-	-
mAb 15B8	+	-	-	-

25 To map the antigenic region on CD40, the four extracellular domains of CD40 were cloned and expressed in insect cells as GST fusion proteins. The secretion of the four domains was ensured with a GP67 secretion signal. Insect cell supernatant was

analyzed by SDS-PAGE and western blot analysis to identify the domain containing the epitope.

Monoclonal antibody 12.12 recognizes an epitope on Domain 2 under both reducing and non-reducing conditions (Table 16; blots not shown). In contrast, monoclonal antibody 5.9 exhibits very weak recognition to Domain 2 (Table 16; blots not shown). Neither of these antibodies recognize Domains 1, 3, or 4 in this analysis.

Table 16. Domain 2 analysis.

	Reduced	Non-reduced
mAb 12.12	++	+++
mAb 5.9	+	+

10

To define more precisely the epitope recognized by mAb 12.12, peptides were synthesized from the extracellular Domain 2 of CD40, which corresponds to the sequence PCGESEFLDTWNRETHCHQHKYCDPNLGLRVQQKGTSETDTICT (residues 61-104 of the sequence shown in SEQ ID NO:10 or SEQ ID NO:12). SPOTs membranes (Sigma) containing thirty-five 10mer peptides with a 1-amino-acid offset were generated. Western blot analysis with mAb 12.12 and anti-human IgG beta-galactosidase as secondary antibody was performed. The blot was stripped and reprobed with mAb 5.9 to determine the region recognized by this antibody

SPOTs analysis probing with anti-CD40 monoclonal antibody 12.12 at 10 µg/ml yielded positive reactions with spots 18 through 22. The sequence region covered by these peptides is shown in Table 17.

Table 17. Results of SPOTs analysis probing with anti-CD40 monoclonal antibody 12.12.

Spot Number	Sequence Region
18	HQHKYCDPNL (residues 78-87 of SEQ ID NO:10 or SEQ ID NO:12)
19	QHKEYCDPNLG (residues 79-88 of SEQ ID NO:10 or SEQ ID NO:12)
20	HKYCDPNLGL (residues 80-89 of SEQ ID NO:10 or SEQ ID NO:12)
21	KYCDPNLGLR (residues 81-90 of SEQ ID NO:10 or SEQ ID NO:12)
22	YCDPNLGLRV (residues 82-91 of SEQ ID NO:10 or SEQ ID NO:12)

- 5 These results correspond to a linear epitope of: YCDPNL (residues 82-87 of the sequence shown in SEQ ID NO:10 or SEQ ID NO:12). This epitope contains Y82, D84, and N86, which have been predicted to be involved in the CD40-CD40 ligand interaction.

- SPOTs analysis with mAb 5.9 showed a weak recognition of peptides represented by spots 20-22 shown in Table 18, suggesting involvement of the region YCDPNLGL (residues 82-89 of the sequence shown in SEQ ID NO:10 or SEQ ID NO:12) in its binding to CD40. It should be noted that the mAbs 12.12 and 5.9 compete with each other for binding to CD40 in BIACORE analysis.

Table 18. Results of SPOTs analysis probing with anti-CD40 monoclonal antibody 5.9.

Spot Number	Sequence Region
20	HKYCDPNLGL (residues 80-89 of SEQ ID NO:10 or SEQ ID NO:12)
21	KYCDPNLGLR (residues 81-90 of SEQ ID NO:10 or SEQ ID NO:12)
22	YCDPNLGLRV (residues 82-91 of SEQ ID NO:10 or SEQ ID NO:12)

- 15 The linear epitopes identified by the SPOTs analyses are within the CD40 B1 module. The sequence of the CD40 B1 module is:

HKYCDPNLGLRVQQKGTSETDTIC (residues 80-103 of SEQ ID NO:10 or SEQ ID NO:12).

Within the linear epitope identified for 12.12 is C83. It is known that this cysteine residue forms a disulphide bond with C103. It is likely that the conformational epitope of the 12.12 mAb contains this disulfide bond (C83-C103) and/or surrounding amino acids conformationally close to C103.

Example 18: Number of CD20 and CD40 Molecules on Namalwa and Daudi Cells

The number of CD20 and CD40 molecules on Namalwa and Daudi cells was determined as outlined in Figure 7, using antibody concentrations of 0.01, 0.1, 1, 10, and 100 µg/ml. As can be seen in Figure 7, the average number of CD20 molecules (target for rituximab) is greater on both the Namalwa and Daudi cell lines than is the number of CD40 molecules (target for mAb 12.12).

Example 19: ADCC of mAb 12.12 and Rituximab Against Daudi Lymphoma Cells

The rituximab and candidate mAb 12.12 were tested *in vitro* for ADCC activity at variable concentrations against lymphoma cell line Daudi as target (T) cells and purified NK cells from healthy human volunteers as effector (E) cells. Freshly isolated human NK cells were mixed with calcein-labeled Daudi lymphoma cells at an E:T ratio of 10. The cell mixture was incubated for 4 hours at 37°C in the presence of the stated concentrations of either mAb 12.12 or rituximab. The calcein level released from lysed target cells in the supernatant was measured as Arbitrary Fluorescent Units (AFU). The percent specific lysis was calculated as: $100 \times (\text{AFU test} - \text{AFU spontaneous release}) / (\text{AFU maximal release} - \text{AFU spontaneous release})$, where AFU spontaneous release is the calcein released by target cells in the absence of antibody or NK cells, and AFU maximal release is the calcein released by target cells upon lysis by detergent.

Antibody concentration-dependent Daudi cell lysis was observed (Figure 8; Table 19 below). The maximum specific lysis of target lymphoma cells induced by anti-CD40 mAb was greater compared to the lysis induced by rituximab (63.6% versus 45.9%, n=6; paired *t* test of mAb 12.12 versus rituximab, p=0.0002). In addition, ED50 for rituximab

was on average (n=6) 51.6 pM, 13-fold higher than ED50 for the anti-CD40 mAb 12.12 for this activity.

Table 19. Comparative ADCC of mAb 12.12 and rituximab against Daudi lymphoma cells.

NK Cell Donor	Maximal Killing (%)		ED50 (pM)	
	mAb 12.12	Rituximab	mAb 12.12	Rituximab
1	50.2	34.9	3.2	14.2
2	83.1	68.6	2.2	27.2
3	64.2	36.9	4.1	66.9
4	53.3	39.5	2.4	47.6
5	74.8	56.6	2.8	24.1
6	56.2	38.9	7.9	129.5
Average	63.6	45.9	3.8	51.6

Example 20: Agonistic and Antagonistic Activity Against Primary Cancer Cell from NHL, CLL, and MM Patients

- 10 In collaboration with clinical investigators, the candidate mAbs is tested for a variety of activities (listed below) against primary cancer cells from NHL and CLL and multiple myeloma patients.
- Agonistic effect in proliferation assays (8 NHL patients, 8 CLL patients and 8 MM patients)
 - 15 • Antagonistic effect in proliferation assays (8 NHL patients, 8 CLL patients and 8 MM patients)
 - Apoptotic effect by Annexin V assay (3-4 NHL patients, 4 CLL patients, and 4 MM patients)
 - Reversing survival signal by Annexin V assay (3 NHL patients, 3 CLL patients and 3 MM patients)
 - 20 • Complement dependent cytotoxicity (4 NHL patients, 4 CLL patients and 4MM patients)

- Antibody dependent cytotoxicity (6 NHL patients, 6 CLL patients and 6 MM patients)

Example 21: Identification of Relevant Animal Species for Toxicity Studies

5 As these two candidate antibodies do not cross-react with rodent CD40, other species must be identified for testing toxicologic effects.

 The ability of the two candidate anti-CD40 antibodies to cross-react with animal CD40 is tested by flow cytometric assays. Rat, rabbit, dog, cynomolgous monkeys and marmoset monkeys are tested for this study.

10 The candidate antibodies show antagonistic activity upon binding to CD40 on human B cells. To identify an animal species that has similar response to candidate antibodies, lymphocytes from species that show binding to candidate antibodies are tested in proliferation assays for antagonistic activity. The lymphocytes from the species selected for antagonistic binding of candidate antibodies are further tested for their ability

15 to serve as effector cells for killing CD40-expressing lymphoma cell lines through the mechanism of ADCC. Finally the selected animal species are tested in an IHC study for the tissue-binding pattern of candidate antibodies. The animal species responding to the candidate antibodies in these assays in a manner similar to that observed for human cells are chosen for toxicology studies.

20 Initial studies indicate that the candidate anti-CD40 mAbs cross-react with cynomolgous monkey CD40.

Example 22: Tumor Targeting Profile of 5.9 and 12.12

 To determine the relative tumor targeting profile of 12.12 and 5.9 mAbs,

25 fluorescent-labeled candidate mAbs and isotype control antibodies are administered into tumor-bearing mice. Tumor specimens and normal organs are harvested at different time points after dosing. The accumulation of labeled antibody in tumors and normal organs is analyzed.

Example 23: Mechanism of Action of 5.9 and 12.12

To elucidate the mechanism(s) that mediates the tumor growth inhibition by the 5.9 and 12.12 mAbs, the following studies are undertaken:

5 Fc-receptor knock-out or blockage model: ADCC is mediated by binding of effector cells such as NK, macrophage, and monocytes to the Fc portion of antibody through Fc receptor. Mice deficient in activating Fc receptors as well as antibodies engineered to disrupt Fc binding to those receptors will block the ADCC mediated tumor growth inhibition. Loss or significantly reduced tumor inhibition in this model will suggest that the tumor growth inhibition by these two candidate mAbs is mainly mediated
10 by ADCC mechanism.

Example 24: Clinical Studies with 5.9 and 12.12

Clinical Objectives

The overall objective is to provide an effective therapy for B cell tumors by
15 targeting them with an anti-CD40 IgG1. These tumors include B-cell lymphoma, Chronic Lymphocytic Lymphoma (CLL), Acute Lymphoblastic Leukemia (ALL), Multiple Myeloma (MM), Waldenstrom's Macroglobulinemia, and Systemic Castleman's Disease. The signal for these diseases is determined in phase II although some measure of activity may be obtained in phase I. Initially the agent is studied as a single agent, but will be
20 combined with other agents, chemotherapeutics, and other antibodies, as development proceeds.

Phase I

- Evaluate safety and pharmacokinetics – dose escalation in subjects with B cell malignancies.
- 25 • Choose dose based on safety, tolerability, and change in serum markers of CD40. In general an MTD is sought but other indications of efficacy (depletion of CD40+ B cells, etc.) may be adequate for dose finding.

- Consideration of more than one dose especially for different indications, e.g., the CLL dose may be different than the NHL. Thus, some dose finding may be necessary in phase II.
- Patients are dosed weekly with real-time pharmacokinetic (Pk) sampling. Initially a 4-week cycle is the maximum dosing allowed. The Pk may be highly variable depending on the disease studied, density of CD40 etc.
- This trial(s) is open to subjects with B-cell lymphoma, CLL, and potentially other B cell malignancies.
- Decision to discontinue or continue studies is based on safety, dose, and preliminary evidence of anti-tumor activity.
- Activity of drug as determined by response rate is determined in Phase II.
- Identify dose(s) for Phase II.

Phase II

- Several trials will be initiated in the above-mentioned tumor types with concentration on B-cell lymphoma, CLL, and Multiple Myeloma (MM). Separate trials may be required in low grade and intermediate/high grade NHL as CD40 may have a different function depending on the grade of lymphoma. In low-grade disease, CD40 acts more as a survival factor, preventing apoptosis. In higher-grade disease, interruption of CD40 signaling may lead to cell death. More than one dose, and more than one schedule may be tested in a randomized phase II setting.

In each disease, target a population that has failed current standard of care:

- CLL: patients who were resistant to Campath® and chemotherapy.
 - Low grade NHL: Rituxan® or CHOP-R failures
 - Intermediate NHL: CHOP-R failures
 - Multiple Myeloma: Chemotherapy failures
- ✓ Decision to discontinue or continue with study is based on proof of therapeutic concept in Phase II

- ✓ Determine whether surrogate marker can be used as early indication of clinical efficacy
- ✓ Identify doses for Phase III

5 **Phase III**

Phase III will depend on where the signal is detected in phase II, and what competing therapies are considered to be the standard. If the signal is in a stage of disease where there is no standard of therapy, then a single arm, well-controlled study could serve as a pivotal trial. If there are competing agents that are considered standard,
10 then head-to-head studies are conducted.

Many modifications and other embodiments of the inventions set forth herein will
15 come to mind to one skilled in the art to which these inventions pertain having the benefit of the teachings presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the inventions are not to be limited to the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended claims and list of embodiments disclosed
20 herein. Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same
25 extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

THAT WHICH IS CLAIMED:

1. A human monoclonal antibody that is capable of specifically binding to a human CD40 antigen expressed on the surface of a human CD40-expressing cell, said
5 monoclonal antibody being free of significant agonistic activity, whereby, when said monoclonal antibody binds to the CD40 antigen expressed on the surface of said cell, the growth or differentiation of said cell is inhibited, wherein said antibody is the monoclonal antibody 5.9 or 12.12.

ANTAGONIST ANTI-CD40 MONOCLONAL ANTIBODIES
AND METHODS FOR THEIR USE

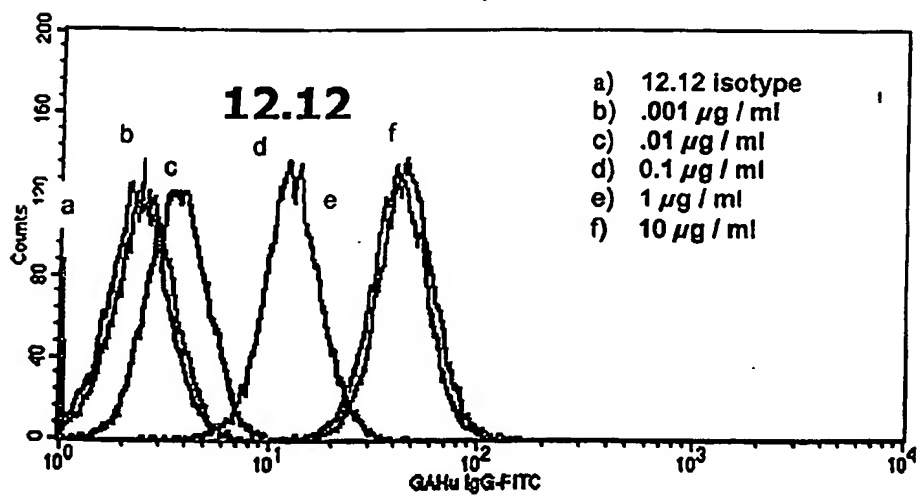
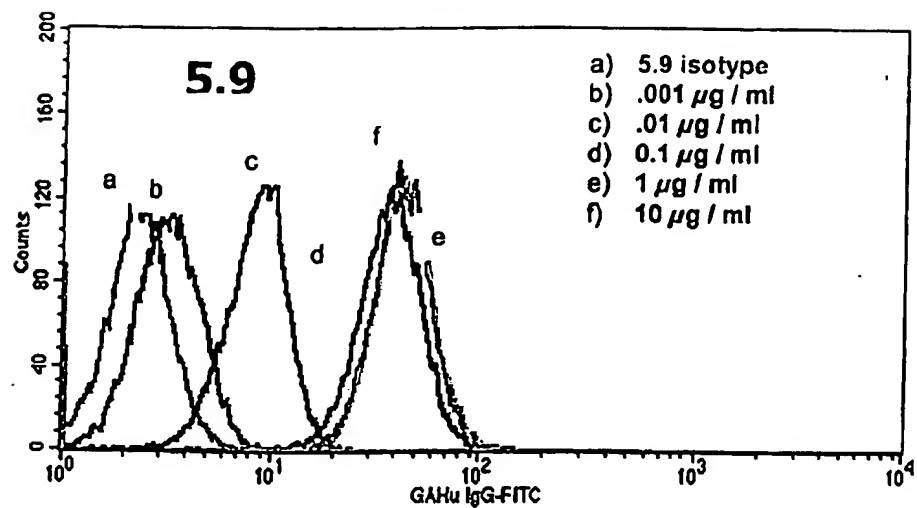
5

ABSTRACT OF THE DISCLOSURE

Methods of therapy for treating diseases mediated by stimulation of CD40 signaling on CD40-expressing cells are provided. The methods comprise administering a therapeutically effective amount of an antagonist anti-CD40 antibody or antigen-binding fragment thereof to a patient in need thereof. The antagonist anti-CD40 antibody or
10 antigen-binding fragment thereof is free of significant agonist activity, but exhibits antagonist activity when the antibody binds a CD40 antigen on a human CD40-expressing cell. Antagonist activity of the anti-CD40 antibody or antigen-binding fragment thereof beneficially inhibits proliferation and/or differentiation of human CD40-expressing cells, such as B cells.

15

FIG. 1A



Fluorescence intensity

FIG. 1B

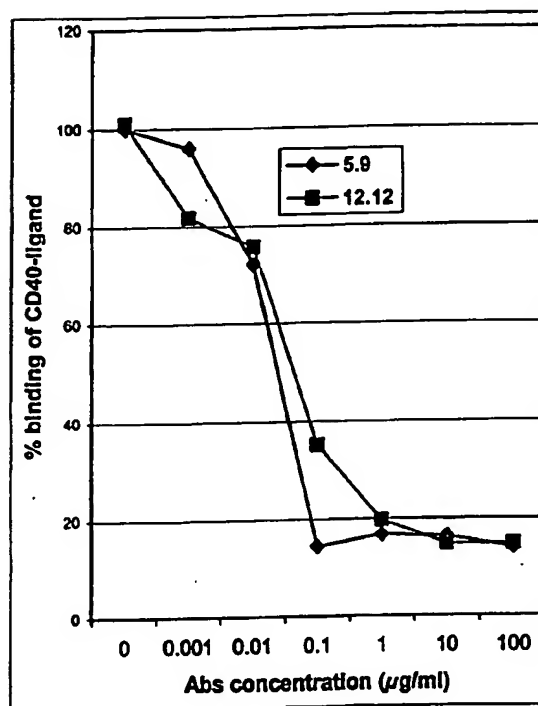


FIG. 2A

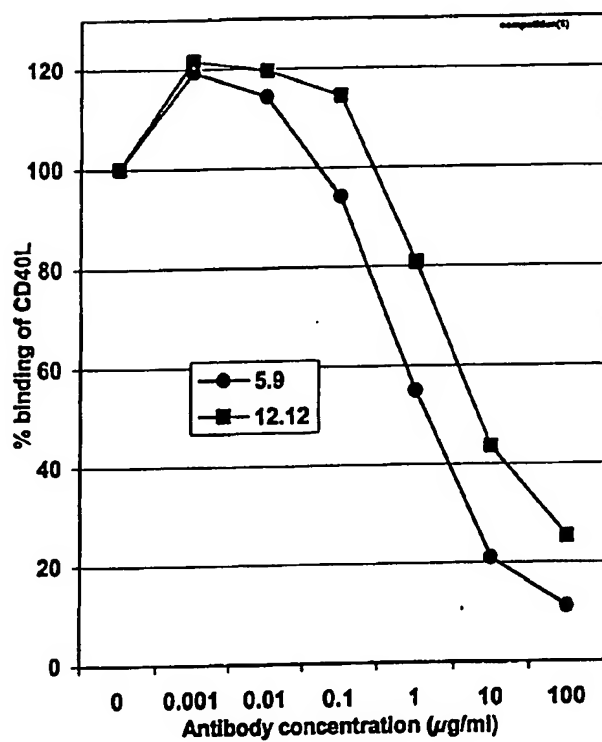


FIG. 2B

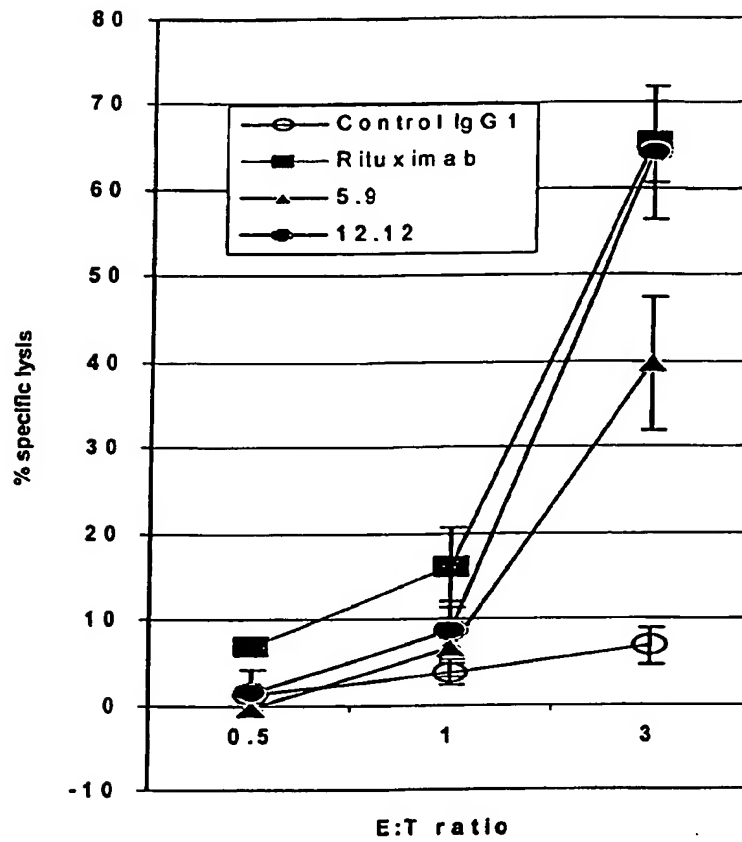


FIG. 3A

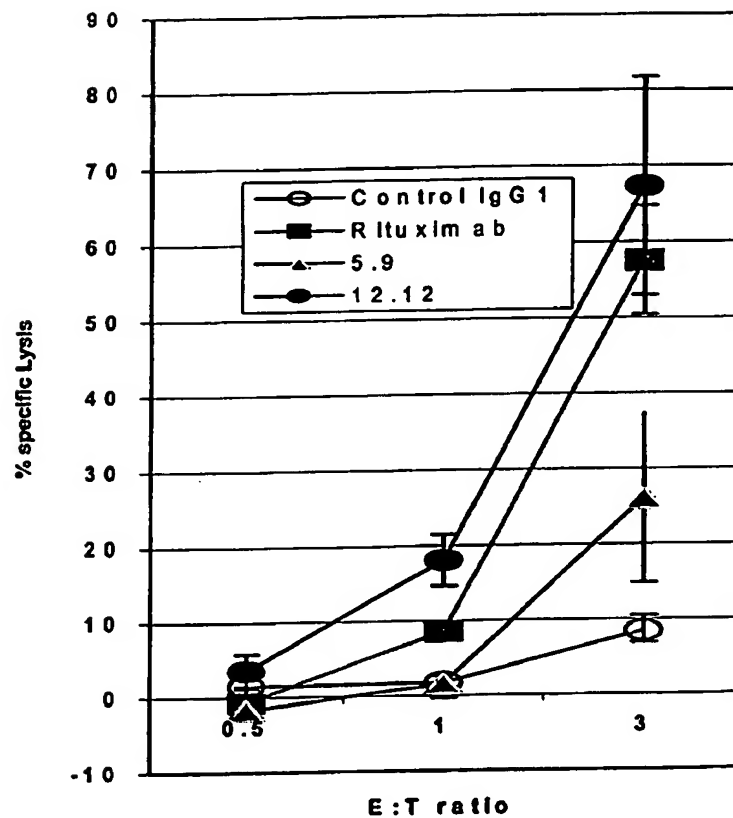


FIG. 3B

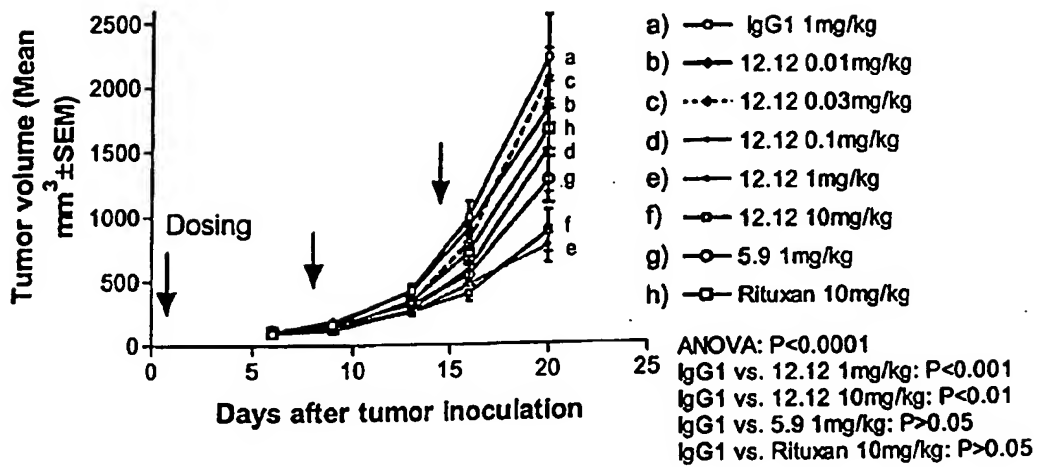


FIG. 4

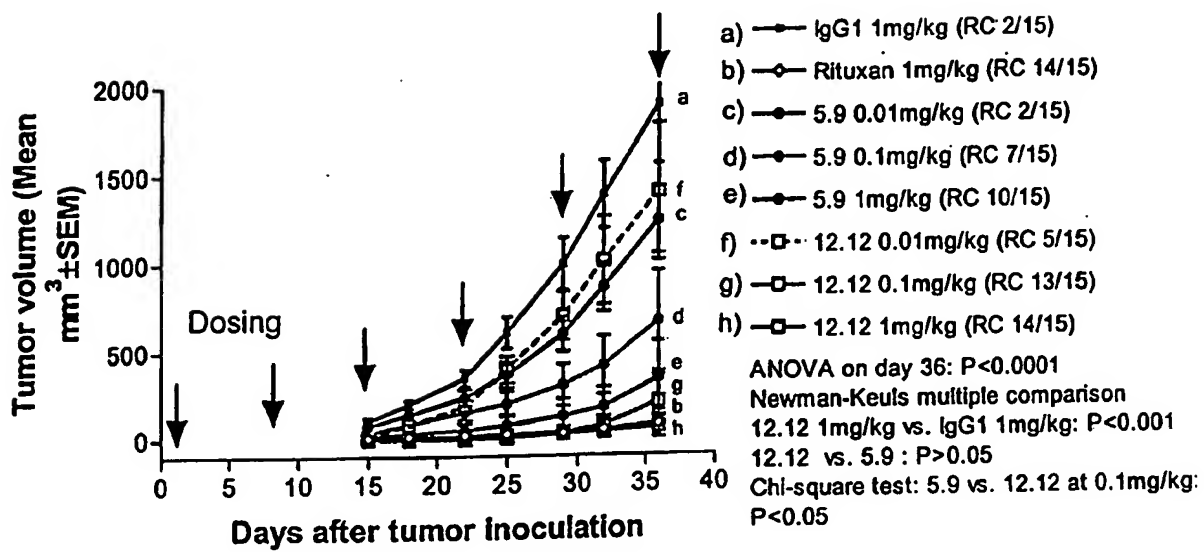


FIG. 5

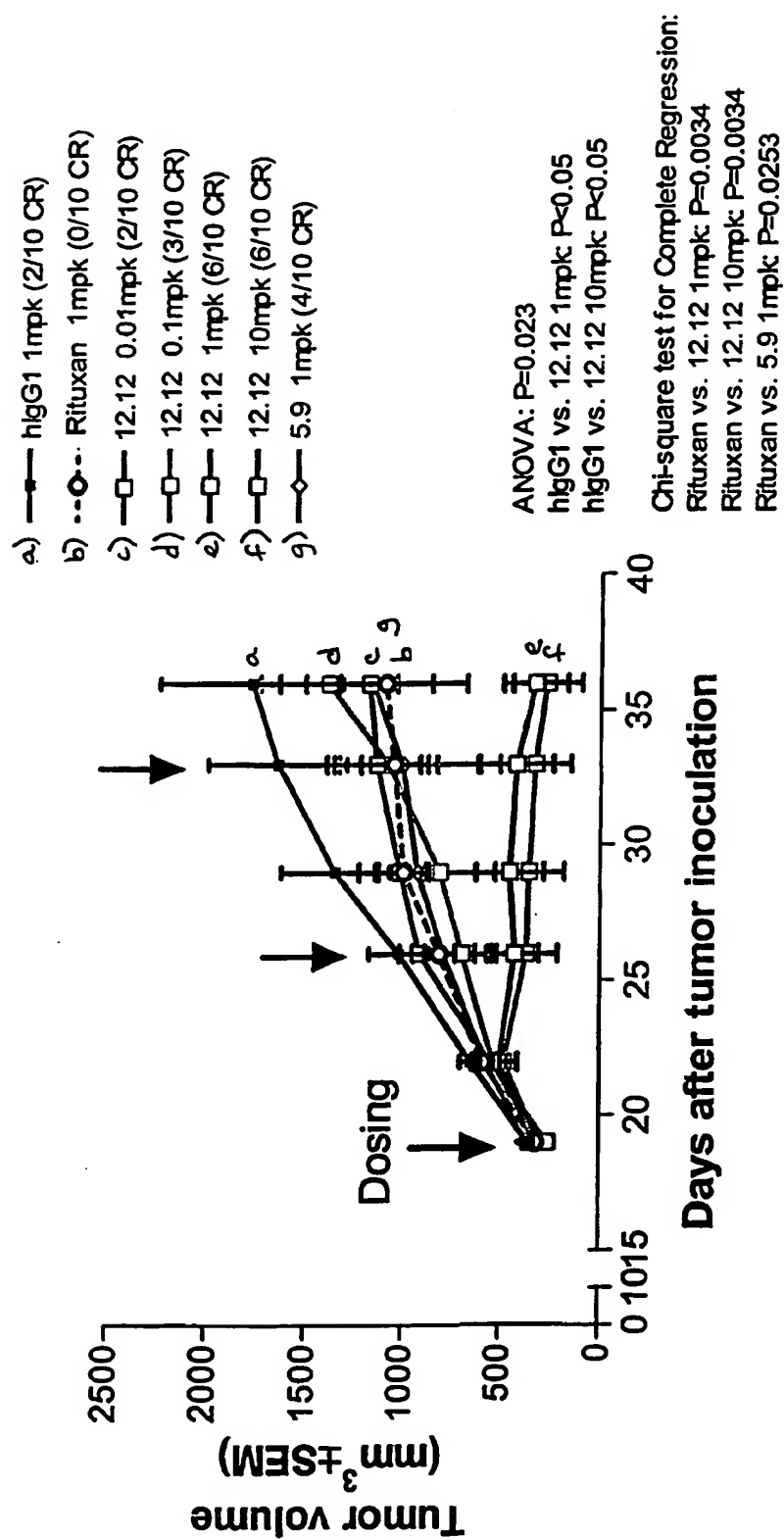


FIG. 6

[illegible]

	Daudii		Namalwa	
Exp.	CD40	CD20	CD40	CD20
E090403	14403.0	93676.5	3296.4	6200.1
E091003	13214.9	108438.5	3081.5	4788.2
E091103	13702.6	100509.1	3165.7	3988.3
E091203	13278.9	128158.3	3164.9	4618.0
Average	13,649.9	107,695.6	3,177.1	4,898.7
Stdev	546.7	14915.9	88.8	933.4

FIG. 7

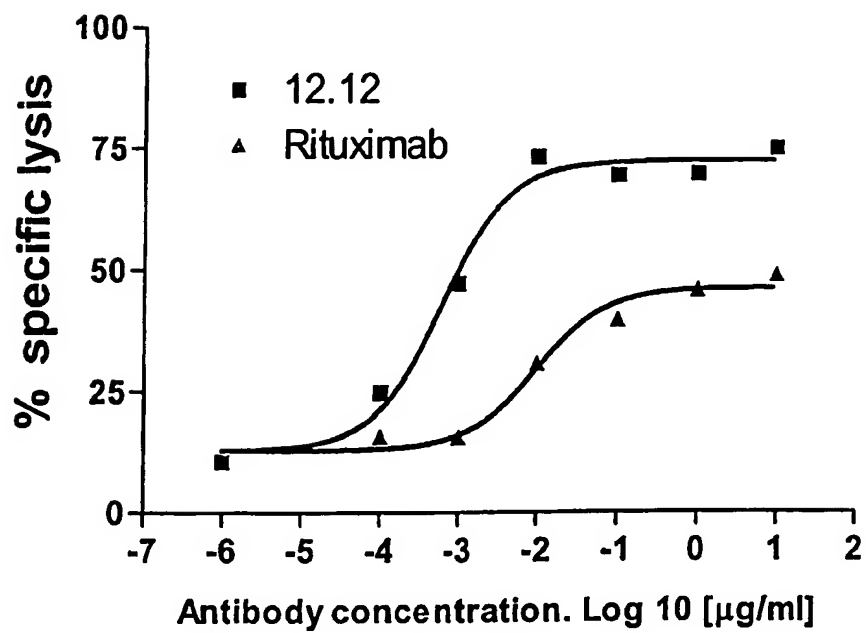


FIG. 8

FIG. 9A

Amino Acid Sequences 12.12

12.12 Light chain:

leader: MALPAQLLGLLMLWVSGSSG

variable:

DIVMTQSPLSLTVPGEPAISCRSSQSLLYSNGYNYLDWYLQKPGQSPQVLIISLGSNR
ASGVPDFRFSGSGSGTDFTLKISRVEAEDVGYYCMQARQTPFTFGPGTKVDIR

constant:

RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDYSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC*

FIG. 9B

12.12 Heavy chain:

leader: MEFGLSWVFLVAILRGVQC

variable:

QVQLVESGGGVVQPGRSRLRLSCAASGFTFSSYGMHWVRQAPGKGLEWVAVISYEESNRY
HADSVKGRFTISRDNKITYLQMNSLRTEDTAVYYCARDGGIAAPGPDYWGQGTILTV
SS

constant:

ASTKGPSVFPLAPASKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQS
SGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELL
GGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE
QYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPP
SREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTV
DKSRWQQGNVFSQSVMEALHNHYTQKSLSLSPGK*

or

alternative constant region:

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQS
SGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELL
GGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE
QYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPP
SREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTV
DKSRWQQGNVFSQSVMEALHNHYTQKSLSLSPGK*

FIG. 10A

Amino Acid Sequence for 5.9
5.9 Light chain:

leader: MALLAQLLGLLMLWVPGSSG

variable:

AIVMTQPPLSSPVTLGQPASISCRSSQSLVHSDGNTYLNWLQQRPGQPPRLLIYKFFRR

LSGVPDRFSGSGAGTDFTLKISRVEAEDVGVIYCMQVTQFPHTFGQGTRLEIK

constant: RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG

NSQESVTEQDSKDSSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC*

FIG. 10B

5.9 Heavy chain:

leader: MGSTAILALLLAVLQGVCA

variable: EVQLVQSGAEVKKPGESLKISCKSGYSFTSYWIGWVRQMPGKGLEWMGI

IYPGDS DTRYSPSFQGGVTISADKSISTAYLQWSSLKASDTAMYICARGTAAGRDIYY

YGMDVWGQGTTVTVSS

constant region:

ASTKGPSVFPLAPASKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQS

SGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELL

GGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE

QYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPP

SREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTV

DKSRWQQGNVFSFSVMHEALHNHYTQKSLSLSPGK*

or alternative constant region:

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQS

SGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELL

GGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE

QYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPP

SREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTV

DKSRWQQGNVFSFSVMHEALHNHYTQKSLSLSPGK

FIG. 11A

DNA sequence of Light chain of 12.12

5'atggcgctccctgctcagctcctggggctgctaagctctgggtctctggatccagtggggatattgtgatgactcagctccac
tctccctgaccgtacccctggagagccggcctccatctcctgcaggtccagtcagagcctcctgtatagtaatggatacaactat
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ctcgcccgctcacaagagcttcaacaggggagagtgtag3'

FIG. 11B

DNA sequence of Heavy chain of 12.12 (including introns)

5'atggagtttgggctgagctgggttttcttctgttctatitaaagggtgccagtgctcaggtgcagttggtggagtctgggggag
gctgtgtccagcctgggaggtccctgagactctcctgtgcagcctctggattcaccttcagtagctatggcatgcactgggtccg
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agagcaggtggcagcaggggaaagcttctcatgctcctgtagcatgaggctctgcacaaccactacacgcagaagagcctc
tccctgtctccgggtaaatga3'

FIG. 12A

Coding sequence for short isoform of human CD40:

```
1 atggttcgtc tgccctcgca gtgcgtcctc tggggctgct tgcgaccgc tgcctatcca
61 gaaccaccca ctgcatgcag agaaaaacag tacctaataa acagtcaagt ctgttctttg
121 tgccagccag gacagaaact ggtgagtgac tgcacagagt tcaatgaaac ggaatgcctt
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481 tgcaccctt ggacaaggc cccaggatcg gctgagagcc ctggtggtga tcccatcat
541 ctccgggac ctgtttgcca tcctcttgt gctggtctt atcaaaaagg tggccaagaa
601 gccaccaat aa
```

FIG. 12B

Encoded short isoform of human CD40:

```
1 mvrplqcvl wgclltavhp epptacrekq ylinsqccsl cpggqklvsd ctefteteci
61 pcgesefldt wnrethchqh kydpnlglr vqqkgtseld tictceegwh ctseacescv
121 lhrscspgfg vkqiatgvsd ticepcpvgf fsnvssafek chpwtrspgs aespaggdphh
181 lrdpvchplg aglyqkggqe anq
```

FIG. 12C

Coding sequence for long isoform of human CD40:

```
1 atggttcgtc tgccctcgca gtgcgtcctc tggggctgct tgctgaccgc tgtccatcca
61 gaaccaccca ctgcatgcag agaaaaacag tacctaataa acagtcagtg ctgttctttg
121 tgccagccag gacagaaact ggtgagtgac tgcacagagt tcactgaaac ggaatgcctt
181 ccttgcggtg aaagcgaatt cctagacacc tggaaacagag agacacactg ccaccagcac
241 aaatactgcg accccaacct agggcttcgg gtccagcaga agggcacctc agaaacagac
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421 accatctgcg agccctgccc agtcgggttc ttctccaatg tgcactctgc ttctgaaaaa
481 tgcaccctt ggacaagctg tgagacaaa gacctggttg tgcaacaggc aggcacaaac
541 aagactgatg ttgtctgtgg tccccaggat cggctgagag ccctggtggt gatccccatc
601 atctcggga tctgtttgc catcctcttg gtgctggtct ttatcaaaaa ggtggccaag
661 aagccaacca ataaggcccc ccacccaag caggaacccc aggagatcaa ttctccgac
721 gatcttctg gctccaacac tgctgctcca gtgcaggaga cttacatgg atgccaaccg
781 gtcaaccagg aggatggcaa agagagtgc atctcagtc aggagagaca gtga
```

FIG. 12D

Encoded long isoform of human CD40:

```
1 mvrplqcvl wgclltavhp epptacrekq ylinsqccsl cpggqklvsd ctefteteci
61 pcgesefldt wnrethchqh kydpnlgr vqqkgtsed tictceegwh ctseacescv
121 lhrscspgfg vkqiatgvsd ticepcpvf fsnvssafek chpwtsctek dlrvqqagtn
181 ktdvvcgpgd rlrlvvipi ifgilfail vlvfikkvak kptnkaphpk qepqeinfpd
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SEQUENCE LISTING

<110> Long, Li
Lugman, Mohammad
Yabannavar, Asha
Zaror, Isabel

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Antibodies and Methods for Their Use

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<400> 1
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Met Ala Leu Pro Ala Gln Leu Leu Gly Leu Leu Met Leu Trp Val Ser
1 5 10 15

gga tcc agt ggg gat att gtg atg act cag tct cca ctc tcc ctg acc 96
Gly Ser Ser Gly Asp Ile Val Met Thr Gln Ser Pro Leu Ser Leu Thr
20 25 30

gtc acc cct gga gag ccg gcc tcc atc tcc tgc agg tcc agt cag agc 144
Val Thr Pro Gly Glu Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser
35 40 45

ctc ctg tat agt aat gga tac aac tat ttg gat tgg tac ctg cag aag 192
Leu Leu Tyr Ser Asn Gly Tyr Asn Tyr Leu Asp Trp Tyr Leu Gln Lys
50 55 60

cca ggg cag tct cca cag gtc ctg atc tct ttg ggt tct aat cgg gcc 240
Pro Gly Gln Ser Pro Gln Val Leu Ile Ser Leu Gly Ser Asn Arg Ala
65 70 75 80

tcc ggg gtc cct gac agg ttc agt ggc agt gga tca ggc aca gat ttt 288
Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe
85 90 95

aca ctg aaa atc agc aga gtg gag gct gag gat gtt ggg gtt tat tac 336
Thr Leu Lys Ile Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr

100	105	110	
tgc atg caa gct cga caa act cca ttc act ttc ggc cct ggg acc aaa			384
Cys Met Gln Ala Arg Gln Thr Pro Phe Thr Phe Gly Pro Gly Thr Lys			
115	120	125	
gtg gat atc aga cga act gtg gct gca cca tct gtc ttc atc ttc ccg			432
Val Asp Ile Arg Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro			
130	135	140	
cca tct gat gag cag ttg aaa tct gga act gcc tct gtt gtg tgc ctg			480
Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu			
145	150	155	160
ctg aat aac ttc tat ccc aga gag gcc aaa gta cag tgg aag gtg gat			528
Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp			
165	170	175	
aac gcc ctc caa tcg ggt aac tcc cag gag agt gtc aca gag cag gac			576
Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp			
180	185	190	
agc aag gac agc acc tac agc ctc agc agc acc ctg acg ctg agc aaa			624
Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys			
195	200	205	
gca gac tac gag aaa cac aaa gtc tac gcc tgc gaa gtc acc cat cag			672
Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln			
210	215	220	
ggc ctg agc tcg ccc gtc aca aag agc ttc aac agg gga gag tgt tag			720
Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys *			
225	230	235	

<210> 2

<211> 239

<212> PRT

<213> Artificial Sequence

<220>

<223> Light chain of 12.12 human anti-CD40 antibody

<400> 2

Met	Ala	Leu	Pro	Ala	Gln	Leu	Leu	Gly	Leu	Leu	Met	Leu	Trp	Val	Ser
1				5				10						15	
Gly	Ser	Ser	Gly	Asp	Ile	Val	Met	Thr	Gln	Ser	Pro	Leu	Ser	Leu	Thr
			20					25					30		
Val	Thr	Pro	Gly	Glu	Pro	Ala	Ser	Ile	Ser	Cys	Arg	Ser	Ser	Gln	Ser
		35					40				45				
Leu	Leu	Tyr	Ser	Asn	Gly	Tyr	Asn	Tyr	Leu	Asp	Trp	Tyr	Leu	Gln	Lys
	50				55				60						
Pro	Gly	Gln	Ser	Pro	Gln	Val	Leu	Ile	Ser	Leu	Gly	Ser	Asn	Arg	Ala
65				70				75					80		
Ser	Gly	Val	Pro	Asp	Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Phe
			85					90					95		

Thr	Leu	Lys	Ile	Ser	Arg	Val	Glu	Ala	Glu	Asp	Val	Gly	Val	Tyr	Tyr	
			100					105					110			
Cys	Met	Gln	Ala	Arg	Gln	Thr	Pro	Phe	Thr	Phe	Gly	Pro	Gly	Thr	Lys	
		115					120					125				
Val	Asp	Ile	Arg	Arg	Thr	Val	Ala	Ala	Pro	Ser	Val	Phe	Ile	Phe	Pro	
	130					135					140					
Pro	Ser	Asp	Glu	Gln	Leu	Lys	Ser	Gly	Thr	Ala	Ser	Val	Val	Cys	Leu	
145					150					155					160	
Leu	Asn	Asn	Phe	Tyr	Pro	Arg	Glu	Ala	Lys	Val	Gln	Trp	Lys	Val	Asp	
			165						170					175		
Asn	Ala	Leu	Gln	Ser	Gly	Asn	Ser	Gln	Glu	Ser	Val	Thr	Glu	Gln	Asp	
		180						185					190			
Ser	Lys	Asp	Ser	Thr	Tyr	Ser	Leu	Ser	Ser	Thr	Leu	Thr	Leu	Ser	Lys	
	195					200					205					
Ala	Asp	Tyr	Glu	Lys	His	Lys	Val	Tyr	Ala	Cys	Glu	Val	Thr	His	Gln	
	210					215					220					
Gly	Leu	Ser	Ser	Pro	Val	Thr	Lys	Ser	Phe	Asn	Arg	Gly	Glu	Cys		
225					230					235						

<210> 3

<211> 2016

<212> DNA

<213> Artificial Sequence

<220>

<223> Coding sequence for heavy chain of 12.12 human
anti-CD40 antibody (with introns)

<400> 3

atg	gag	ttt	ggg	ctg	agc	tgg	gtt	ttc	ctt	ggt	gct	att	tta	aga	ggc	48
gtc	cag	tgt	cag	gtg	cag	ttg	gtg	gag	tct	ggg	gga	ggc	gtg	gtc	cag	96
cct	ggg	agg	tcc	ctg	aga	ctc	tcc	tgt	gca	gcc	tct	gga	ttc	acc	ttc	144
agt	agc	tat	ggc	atg	cac	tgg	gtc	cgc	cag	gct	cca	ggc	aag	ggg	ctg	192
gag	tgg	gtg	gca	gtt	ata	tca	tat	gag	gaa	agt	aat	aga	tac	cat	gca	240
gac	tcc	gtg	aag	ggc	cga	ttc	acc	atc	tcc	aga	gac	aat	tcc	aag	atc	288
acg	ctg	tat	ctg	caa	atg	aac	agc	ctc	aga	act	gag	gac	acg	gct	gtg	336
tat	tac	tgt	gcg	aga	gat	ggg	ggg	ata	gca	gca	cct	ggg	cct	gac	tac	384
tgg	ggc	cag	gga	acc	ctg	gtc	acc	gtc	tcc	tca	gca	agt	acc	aag	ggc	432
cca	tcc	gtc	ttc	ccc	ctg	gcg	ccc	gct	agc	aag	agc	acc	tct	ggg	ggc	480
aca	gcg	gcc	ctg	ggc	tgc	ctg	gtc	aag	gac	tac	ttc	ccc	gaa	ccg	gtg	528
acg	gtg	tcg	tgg	aac	tca	ggc	gcc	ctg	acc	agc	ggc	gtg	cac	acc	ttc	576
ccg	gct	gtc	cta	cag	tcc	tca	gga	ctc	tac	tcc	ctc	agc	agc	gtg	gtg	624
acc	gtg	ccc	tcc	agc	agc	ttg	ggc	acc	cag	acc	tac	atc	tgc	aac	gtg	672
aat	cac	aag	ccc	agc	aac	acc	aag	gtg	gac	aag	aga	gtt	ggg	gag	agg	720
cca	gca	cag	gga	ggg	agg	gtg	tct	gct	gga	agc	cag	gct	cag	cgc	tcc	768
tgc	ctg	gac	gca	tcc	cgg	cta	tgc	agt	ccc	agt	cca	ggg	cag	caa	ggc	816
agg	ccc	cgt	ctg	cct	ctt	cac	ccg	gag	gcc	tct	gcc	cgc	ccc	act	cat	864
gct	cag	gga	gag	ggg	ctt	ctg	gct	ttt	tcc	cca	ggc	tct	ggg	cag	gca	912
cag	gct	agg	tgc	ccc	taa	ccc	agg	ccc	tgc	aca	caa	agg	ggc	agg	tgc	960
tgg	gct	cag	acc	tgc	caa	gag	cca	tat	ccg	gga	gga	ccc	tgc	ccc	tga	1008
cct	aag	ccc	acc	cca	aag	gcc	aaa	ctc	tcc	act	ccc	tca	gct	cgg	aca	1056
cct	tct	ctc	ctc	cca	gat	tcc	agt	aac	tcc	caa	tct	tct	ctc	tgc	aga	1104
gcc	caa	atc	ttg	tga	caa	aac	tca	cac	atg	ccc	acc	gtg	ccc	agg	taa	1152
gcc	agc	cca	ggc	ctc	gcc	ctc	cag	ctc	aag	gcg	gga	cag	gtg	ccc	tag	1200
agt	agc	ctg	cat	cca	ggg	aca	ggc	ccc	agc	cgg	gtg	ctg	aca	cgt	cca	1248
cct	cca	tct	ctt	cct	cag	cac	ctg	aac	tcc	tgg	ggg	gac	cgt	cag	tct	1296

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tcc tct tcc ccc caa aac cca agg aca ccc tca tga tct ccc gga ccc 1344
ctg agg tca cat gcg tgg tgg tgg acg tga gcc acg aag acc ctg agg 1392
tca agt tca act ggt acg tgg acg gcg tgg agg tgc ata atg cca aga 1440
caa agc cgc ggg agg agc agt aca aca gca cgt acc gtg tgg tca gcg 1488
tcc tca ccg tcc tgc acc agg act ggc tga atg gca agg agt aca agt 1536
gca agg tct cca aca aag ccc tcc cag ccc cca tcg aga aaa cca tct 1584
cca aag cca aag gtg gga ccc gtg ggg tgc gag ggc cac atg gac aga 1632
ggc cgg ctc ggc cca ccc tct gcc ctg aga gtg acc gct gta cca acc 1680
tct gtc cct aca ggg cag ccc cga gaa cca cag gtg tac acc ctg ccc 1728
cca tcc cgg gag gag atg acc aag aac cag gtc agc ctg acc tgc ctg 1776
gtc aaa ggc ttc tat ccc agc gac atc gcc gtg gag tgg gag agc aat 1824
ggg cag ccg gag aac aac tac aag acc acg cct ccc gtg ctg gac tcc 1872
gac ggc tcc ttc ttc ctc tat agc aag ctc acc gtg gac aag agc agg 1920
tgg cag cag ggg aac gtc ttc tca tgc tcc gtg atg cat gag gct ctg 1968
cac aac cac tac acg cag aag agc ctc tcc ctg tct ccg ggt aaa tga 2016

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<210> 4
<211> 469
<212> PRT
<213> Artificial Sequence

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<220>
<223> Heavy chain of 12.12 human anti-CD40 antibody

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<400> 4
Met Glu Phe Gly Leu Ser Trp Val Phe Leu Val Ala Ile Leu Arg Gly
1      5      10      15
Val Gln Cys Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln
20     25     30
Pro Gly Arg Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
35     40     45
Ser Ser Tyr Gly Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
50     55     60
Glu Trp Val Ala Val Ile Ser Tyr Glu Glu Ser Asn Arg Tyr His Ala
65     70     75     80
Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Ile
85     90     95
Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Thr Glu Asp Thr Ala Val
100    105    110
Tyr Tyr Cys Ala Arg Asp Gly Gly Ile Ala Ala Pro Gly Pro Asp Tyr
115    120    125
Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly
130    135    140
Pro Ser Val Phe Pro Leu Ala Pro Ala Ser Lys Ser Thr Ser Gly Gly
145    150    155    160
Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val
165    170    175
Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe
180    185    190
Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
195    200    205
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val
210    215    220
Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys Arg Val Glu Pro Lys
225    230    235    240
Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu

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Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly
 130 135 140
 Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly
 145 150 155 160
 Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val
 165 170 175
 Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe
 180 185 190
 Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
 195 200 205
 Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val
 210 215 220
 Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys Arg Val Glu Pro Lys
 225 230 235 240
 Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu
 245 250 255
 Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr
 260 265 270
 Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val
 275 280 285
 Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val
 290 295 300
 Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser
 305 310 315 320
 Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu
 325 330 335
 Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala
 340 345 350
 Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro
 355 360 365
 Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln
 370 375 380
 Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala
 385 390 395 400
 Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr
 405 410 415
 Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu
 420 425 430
 Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser
 435 440 445
 Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser
 450 455 460
 Leu Ser Pro Gly Lys
 465

<210> 6
 <211> 239
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Light chain of 5.9 human anti-CD40 antibody

<400> 6
 Met Ala Leu Leu Ala Gln Leu Leu Gly Leu Leu Met Leu Trp Val Pro
 1 5 10 15

Gly	Ser	Ser	Gly	Ala	Ile	Val	Met	Thr	Gln	Pro	Pro	Leu	Ser	Ser	Pro	
			20					25					30			
Val	Thr	Leu	Gly	Gln	Pro	Ala	Ser	Ile	Ser	Cys	Arg	Ser	Ser	Gln	Ser	
		35					40					45				
Leu	Val	His	Ser	Asp	Gly	Asn	Thr	Tyr	Leu	Asn	Trp	Leu	Gln	Gln	Arg	
		50				55					60					
Pro	Gly	Gln	Pro	Pro	Arg	Leu	Leu	Ile	Tyr	Lys	Phe	Phe	Arg	Arg	Leu	
65					70					75					80	
Ser	Gly	Val	Pro	Asp	Arg	Phe	Ser	Gly	Ser	Gly	Ala	Gly	Thr	Asp	Phe	
				85					90					95		
Thr	Leu	Lys	Ile	Ser	Arg	Val	Glu	Ala	Glu	Asp	Val	Gly	Val	Tyr	Tyr	
			100					105					110			
Cys	Met	Gln	Val	Thr	Gln	Phe	Pro	His	Thr	Phe	Gly	Gln	Gly	Thr	Arg	
		115					120					125				
Leu	Glu	Ile	Lys	Arg	Thr	Val	Ala	Ala	Pro	Ser	Val	Phe	Ile	Phe	Pro	
		130				135					140					
Pro	Ser	Asp	Glu	Gln	Leu	Lys	Ser	Gly	Thr	Ala	Ser	Val	Val	Cys	Leu	
145					150					155					160	
Leu	Asn	Asn	Phe	Tyr	Pro	Arg	Glu	Ala	Lys	Val	Gln	Trp	Lys	Val	Asp	
			165					170						175		
Asn	Ala	Leu	Gln	Ser	Gly	Asn	Ser	Gln	Glu	Ser	Val	Thr	Glu	Gln	Asp	
			180					185					190			
Ser	Lys	Asp	Ser	Thr	Tyr	Ser	Leu	Ser	Ser	Thr	Leu	Thr	Leu	Ser	Lys	
		195					200					205				
Ala	Asp	Tyr	Glu	Lys	His	Lys	Val	Tyr	Ala	Cys	Glu	Val	Thr	His	Gln	
		210				215					220					
Gly	Leu	Ser	Ser	Pro	Val	Thr	Lys	Ser	Phe	Asn	Arg	Gly	Glu	Cys		
225					230					235						

<210> 7

<211> 474

<212> PRT

<213> Artificial Sequence

<220>

<223> Heavy chain of 5.9 human anti-CD40 antibody

<400> 7

Met	Gly	Ser	Thr	Ala	Ile	Leu	Ala	Leu	Leu	Leu	Ala	Val	Leu	Gln	Gly	
1				5				10						15		
Val	Cys	Ala	Glu	Val	Gln	Leu	Val	Gln	Ser	Gly	Ala	Glu	Val	Lys	Lys	
			20					25					30			
Pro	Gly	Glu	Ser	Leu	Lys	Ile	Ser	Cys	Lys	Gly	Ser	Gly	Tyr	Ser	Phe	
		35					40					45				
Thr	Ser	Tyr	Trp	Ile	Gly	Trp	Val	Arg	Gln	Met	Pro	Gly	Lys	Gly	Leu	
		50				55					60					
Glu	Trp	Met	Gly	Ile	Ile	Tyr	Pro	Gly	Asp	Ser	Asp	Thr	Arg	Tyr	Ser	
65					70					75					80	
Pro	Ser	Phe	Gln	Gly	Gln	Val	Thr	Ile	Ser	Ala	Asp	Lys	Ser	Ile	Ser	
			85					90						95		
Thr	Ala	Tyr	Leu	Gln	Trp	Ser	Ser	Leu	Lys	Ala	Ser	Asp	Thr	Ala	Met	
			100					105					110			
Tyr	Tyr	Cys	Ala	Arg	Gly	Thr	Ala	Ala	Gly	Arg	Asp	Tyr	Tyr	Tyr	Tyr	
		115					120					125				
Tyr	Gly	Met	Asp	Val	Trp	Gly	Gln	Gly	Thr	Thr	Val	Thr	Val	Ser	Ser	
		130				135					140					

Ala	Ser	Thr	Lys	Gly	Pro	Ser	Val	Phe	Pro	Leu	Ala	Pro	Ala	Ser	Lys
145					150				155						160
Ser	Thr	Ser	Gly	Gly	Thr	Ala	Ala	Leu	Gly	Cys	Leu	Val	Lys	Asp	Tyr
			165						170					175	
Phe	Pro	Glu	Pro	Val	Thr	Val	Ser	Trp	Asn	Ser	Gly	Ala	Leu	Thr	Ser
			180					185					190		
Gly	Val	His	Thr	Phe	Pro	Ala	Val	Leu	Gln	Ser	Ser	Gly	Leu	Tyr	Ser
		195					200					205			
Leu	Ser	Ser	Val	Val	Thr	Val	Pro	Ser	Ser	Ser	Leu	Gly	Thr	Gln	Thr
	210					215					220				
Tyr	Ile	Cys	Asn	Val	Asn	His	Lys	Pro	Ser	Asn	Thr	Lys	Val	Asp	Lys
225					230					235					240
Arg	Val	Glu	Pro	Lys	Ser	Cys	Asp	Lys	Thr	His	Thr	Cys	Pro	Pro	Cys
				245					250					255	
Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gly	Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro
			260					265					270		
Lys	Pro	Lys	Asp	Thr	Leu	Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys
		275					280					285			
Val	Val	Val	Asp	Val	Ser	His	Glu	Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp
	290					295					300				
Tyr	Val	Asp	Gly	Val	Glu	Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu
305					310					315					320
Glu	Gln	Tyr	Asn	Ser	Thr	Tyr	Arg	Val	Val	Ser	Val	Leu	Thr	Val	Leu
			325						330					335	
His	Gln	Asp	Trp	Leu	Asn	Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn
		340						345					350		
Lys	Ala	Leu	Pro	Ala	Pro	Ile	Glu	Lys	Thr	Ile	Ser	Lys	Ala	Lys	Gly
		355					360					365			
Gln	Pro	Arg	Glu	Pro	Gln	Val	Tyr	Thr	Leu	Pro	Pro	Ser	Arg	Glu	Glu
	370					375					380				
Met	Thr	Lys	Asn	Gln	Val	Ser	Leu	Thr	Cys	Leu	Val	Lys	Gly	Phe	Tyr
385				390						395					400
Pro	Ser	Asp	Ile	Ala	Val	Glu	Trp	Glu	Ser	Asn	Gly	Gln	Pro	Glu	Asn
			405					410						415	
Asn	Tyr	Lys	Thr	Thr	Pro	Pro	Val	Leu	Asp	Ser	Asp	Gly	Ser	Phe	Phe
			420					425					430		
Leu	Tyr	Ser	Lys	Leu	Thr	Val	Asp	Lys	Ser	Arg	Trp	Gln	Gln	Gly	Asn
		435					440					445			
Val	Phe	Ser	Cys	Ser	Val	Met	His	Glu	Ala	Leu	His	Asn	His	Tyr	Thr
	450					455					460				
Gln	Lys	Ser	Leu	Ser	Leu	Ser	Pro	Gly	Lys						
465					470										

<210> 8

<211> 474

<212> PRT

<213> Artificial Sequence

<220>

<223> Heavy chain of variant of 5.9 human anti-CD40 antibody

<400> 8

Met	Gly	Ser	Thr	Ala	Ile	Leu	Ala	Leu	Leu	Leu	Ala	Val	Leu	Gln	Gly
1				5				10					15		
Val	Cys	Ala	Glu	Val	Gln	Leu	Val	Gln	Ser	Gly	Ala	Glu	Val	Lys	Lys

<210> 9
 <211> 612
 <212> DNA
 <213> Homo sapiens

<220>
 <221> CDS
 <222> (1)...(612)

<221> misc_feature
 <222> (0)...(0)
 <223> Coding sequence for short isoform of human CD40

<400> 9
 atg gtt cgt ctg cct ctg cag tgc gtc ctc tgg ggc tgc ttg ctg acc 48
 Met Val Arg Leu Pro Leu Gln Cys Val Leu Trp Gly Cys Leu Leu Thr
 1 5 10 15
 gct gtc cat cca gaa cca ccc act gca tgc aga gaa aaa cag tac cta 96
 Ala Val His Pro Glu Pro Pro Thr Ala Cys Arg Glu Lys Gln Tyr Leu
 20 25 30
 ata aac agt cag tgc tgt tct ttg tgc cag cca gga cag aaa ctg gtg 144
 Ile Asn Ser Gln Cys Cys Ser Leu Cys Gln Pro Gly Gln Lys Leu Val
 35 40 45
 agt gac tgc aca gag ttc act gaa acg gaa tgc ctt cct tgc ggt gaa 192
 Ser Asp Cys Thr Glu Phe Thr Glu Thr Glu Cys Leu Pro Cys Gly Glu
 50 55 60
 agc gaa ttc cta gac acc tgg aac aga gag aca cac tgc cac cag cac 240
 Ser Glu Phe Leu Asp Thr Trp Asn Arg Glu Thr His Cys His Gln His
 65 70 75 80
 aaa tac tgc gac ccc aac cta ggg ctt cgg gtc cag cag aag ggc acc 288
 Lys Tyr Cys Asp Pro Asn Leu Gly Leu Arg Val Gln Gln Lys Gly Thr
 85 90 95
 tca gaa aca gac acc atc tgc acc tgt gaa gaa ggc tgg cac tgt acg 336
 Ser Glu Thr Asp Thr Ile Cys Thr Cys Glu Glu Gly Trp His Cys Thr
 100 105 110
 agt gag gcc tgt gag agc tgt gtc ctg cac cgc tca tgc tcg ccc ggc 384
 Ser Glu Ala Cys Glu Ser Cys Val Leu His Arg Ser Cys Ser Pro Gly
 115 120 125
 ttt ggg gtc aag cag att gct aca ggg gtt tct gat acc atc tgc gag 432
 Phe Gly Val Lys Gln Ile Ala Thr Gly Val Ser Asp Thr Ile Cys Glu
 130 135 140
 ccc tgc cca gtc ggc ttc ttc tcc aat gtg tca tct gct ttc gaa aaa 480
 Pro Cys Pro Val Gly Phe Phe Ser Asn Val Ser Ser Ala Phe Glu Lys
 145 150 155 160
 tgt cac cct tgg aca agg tcc cca gga tcg gct gag agc cct ggt ggt 528

Cys	His	Pro	Trp	Thr	Arg	Ser	Pro	Gly	Ser	Ala	Glu	Ser	Pro	Gly	Gly	
				165				170						175		
gat ccc cat cat ctt cgg gat cct gtt tgc cat cct ctt ggt gct ggt																576
Asp	Pro	His	His	Leu	Arg	Asp	Pro	Val	Cys	His	Pro	Leu	Gly	Ala	Gly	
			180					185					190			
ctt tat caa aaa ggt ggc caa gaa gcc aac caa taa																612
Leu	Tyr	Gln	Lys	Gly	Gly	Gln	Glu	Ala	Asn	Gln	*					
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Ile	Asn	Ser	Gln	Cys	Cys	Ser	Leu	Cys	Gln	Pro	Gly	Gln	Lys	Leu	Val	
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Ser	Glu	Phe	Leu	Asp	Thr	Trp	Asn	Arg	Glu	Thr	His	Cys	His	Gln	His	
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Lys	Tyr	Cys	Asp	Pro	Asn	Leu	Gly	Leu	Arg	Val	Gln	Gln	Lys	Gly	Thr	
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Ser	Glu	Thr	Asp	Thr	Ile	Cys	Thr	Cys	Glu	Glu	Gly	Trp	His	Cys	Thr	
			100					105					110			
Ser	Glu	Ala	Cys	Glu	Ser	Cys	Val	Leu	His	Arg	Ser	Cys	Ser	Pro	Gly	
		115					120					125				
Phe	Gly	Val	Lys	Gln	Ile	Ala	Thr	Gly	Val	Ser	Asp	Thr	Ile	Cys	Glu	
	130					135					140					
Pro	Cys	Pro	Val	Gly	Phe	Phe	Ser	Asn	Val	Ser	Ser	Ala	Phe	Glu	Lys	
145					150					155					160	
Cys	His	Pro	Trp	Thr	Arg	Ser	Pro	Gly	Ser	Ala	Glu	Ser	Pro	Gly	Gly	
			165						170					175		
Asp	Pro	His	His	Leu	Arg	Asp	Pro	Val	Cys	His	Pro	Leu	Gly	Ala	Gly	
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gct gtc cat cca gaa cca ccc act gca tgc aga gaa aaa cag tac cta	96
Ala Val His Pro Glu Pro Pro Thr Ala Cys Arg Glu Lys Gln Tyr Leu	
20 25 30	
ata aac agt cag tgc tgt tct ttg tgc cag cca gga cag aaa ctg gtg	144
Ile Asn Ser Gln Cys Cys Ser Leu Cys Gln Pro Gly Gln Lys Leu Val	
35 40 45	
agt gac tgc aca gag ttc act gaa acg gaa tgc ctt cct tgc ggt gaa	192
Ser Asp Cys Thr Glu Phe Thr Glu Thr Glu Cys Leu Pro Cys Gly Glu	
50 55 60	
agc gaa ttc cta gac acc tgg aac aga gag aca cac tgc cac cag cac	240
Ser Glu Phe Leu Asp Thr Trp Asn Arg Glu Thr His Cys His Gln His	
65 70 75 80	
aaa tac tgc gac ccc aac cta ggg ctt cgg gtc cag cag aag ggc acc	288
Lys Tyr Cys Asp Pro Asn Leu Gly Leu Arg Val Gln Gln Lys Gly Thr	
85 90 95	
tca gaa aca gac acc atc tgc acc tgt gaa gaa ggc tgg cac tgt acg	336
Ser Glu Thr Asp Thr Ile Cys Thr Cys Glu Glu Gly Trp His Cys Thr	
100 105 110	
agt gag gcc tgt gag agc tgt gtc ctg cac cgc tca tgc tcg ccc ggc	384
Ser Glu Ala Cys Glu Ser Cys Val Leu His Arg Ser Cys Ser Pro Gly	
115 120 125	
ttt ggg gtc aag cag att gct aca ggg gtt tct gat acc atc tgc gag	432
Phe Gly Val Lys Gln Ile Ala Thr Gly Val Ser Asp Thr Ile Cys Glu	
130 135 140	
ccc tgc cca gtc ggc ttc ttc tcc aat gtg tca tct gct ttc gaa aaa	480
Pro Cys Pro Val Gly Phe Phe Ser Asn Val Ser Ser Ala Phe Glu Lys	
145 150 155 160	
tgt cac cct tgg aca agc tgt gag acc aaa gac ctg gtt gtg caa cag	528
Cys His Pro Trp Thr Ser Cys Glu Thr Lys Asp Leu Val Val Gln Gln	
165 170 175	
gca ggc aca aac aag act gat gtt gtc tgt ggt ccc cag gat cgg ctg	576
Ala Gly Thr Asn Lys Thr Asp Val Val Cys Gly Pro Gln Asp Arg Leu	
180 185 190	
aga gcc ctg gtg gtg atc ccc atc atc ttc ggg atc ctg ttt gcc atc	624
Arg Ala Leu Val Val Ile Pro Ile Ile Phe Gly Ile Leu Phe Ala Ile	
195 200 205	
ctc ttg gtg ctg gtc ttt atc aaa aag gtg gcc aag aag cca acc aat	672
Leu Leu Val Leu Val Phe Ile Lys Lys Val Ala Lys Lys Pro Thr Asn	

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225	230	235	240
gat ctt cct ggc tcc aac act gct gct cca gtg cag gag act tta cat			768
Asp Leu Pro Gly Ser Asn Thr Ala Ala Pro Val Gln Glu Thr Leu His			
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gga tgc caa ccg gtc acc cag gag gat ggc aaa gag agt cgc atc tca			816
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Val Gln Glu Arg Gln *			
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Ile Asn Ser Gln Cys Cys Ser Leu Cys Gln Pro Gly Gln Lys Leu Val			
	35	40	45
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	50	55	60
Ser Glu Phe Leu Asp Thr Trp Asn Arg Glu Thr His Cys His Gln His			
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Lys Tyr Cys Asp Pro Asn Leu Gly Leu Arg Val Gln Gln Lys Gly Thr			
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Ser Glu Thr Asp Thr Ile Cys Thr Cys Glu Glu Gly Trp His Cys Thr			
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Ser Glu Ala Cys Glu Ser Cys Val Leu His Arg Ser Cys Ser Pro Gly			
	115	120	125
Phe Gly Val Lys Gln Ile Ala Thr Gly Val Ser Asp Thr Ile Cys Glu			
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Pro Cys Pro Val Gly Phe Phe Ser Asn Val Ser Ser Ala Phe Glu Lys			
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Cys His Pro Trp Thr Ser Cys Glu Thr Lys Asp Leu Val Val Gln Gln			
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Ala Gly Thr Asn Lys Thr Asp Val Val Cys Gly Pro Gln Asp Arg Leu			
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Arg Ala Leu Val Val Ile Pro Ile Ile Phe Gly Ile Leu Phe Ala Ile			
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Leu Leu Val Leu Val Phe Ile Lys Lys Val Ala Lys Lys Pro Thr Asn			
	210	215	220
Lys Ala Pro His Pro Lys Gln Glu Pro Gln Glu Ile Asn Phe Pro Asp			
	225	230	235
Asp Leu Pro Gly Ser Asn Thr Ala Ala Pro Val Gln Glu Thr Leu His			
	245	250	255

Gly	Cys	Gln	Pro	Val	Thr	Gln	Glu	Asp	Gly	Lys	Glu	Ser	Arg	Ile	Ser
			260					265					270		
Val	Gln	Glu	Arg	Gln											
		275													

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